INSTRUCTION MANUAL

Monarch® HMW DNA Extraction Kit for Tissue

NEB #T3060S/L

5/50 preps

Version 3.0_3/24

Table of Contents

Kit Components

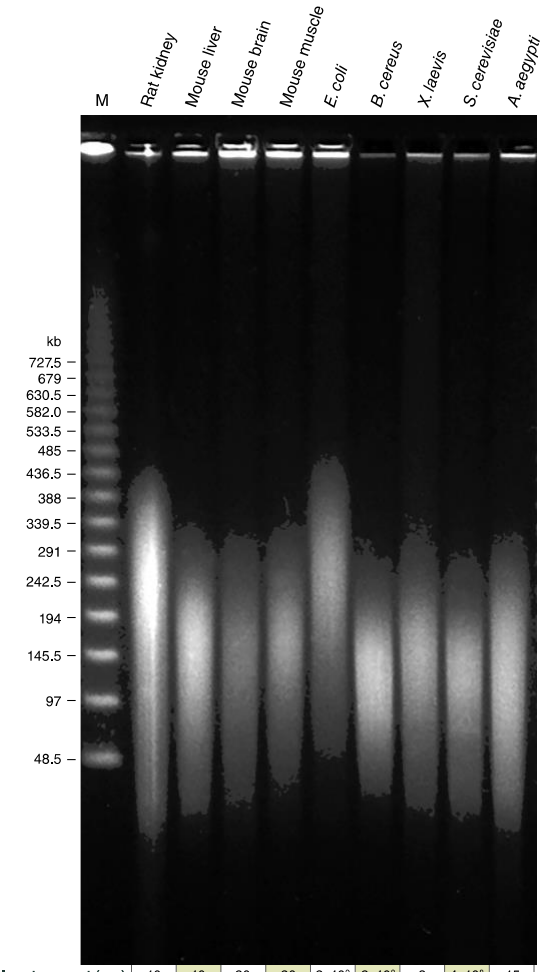
After opening the kit, RNase A and Proteinase K should be stored at -20°C. The remainder of the kit should be stored at room temperature. Always keep buffer bottles tightly closed. For information regarding the composition of buffers, please consult the Safety Data Sheets available on our website (www.neb.com/T3060). Proper laboratory safety practices should be employed, including the use of lab coats, gloves and eye protection.

NEB #		T3060S 5 preps	T3060L 50 preps	STORAGE TEMPERATURE
T3005	Monarch DNA Capture Beads	10 beads	100 beads	25°C
T3004	Monarch Bead Retainers	5 retainers	50 retainers	25°C
T3003	Monarch 2 ml Tubes	10 tubes	100 tubes	25°C
T3002	Monarch Pestles	5 pestles	50 pestles	25°C
T3001	Monarch Pestle Tubes	5 tubes	50 tubes	25°C
T2118	Monarch Spin Collection Tubes	5 tubes	50 tubes	25°C
T3061	Monarch HMW gDNA Tissue Lysis Buffer	3.5 ml	31 ml	25°C
T3062	Monarch Protein Separation Solution	2 ml	18 ml	25°C
T3015	Monarch gDNA Wash Buffer	4.5 ml	18 ml	25°C
T3056	Monarch Elution Buffer II	1.5 ml	12 ml	25°C
T3018	Monarch RNase A	2 x 35 µl	500 µl	-20°C after opening
P8200	Proteinase K, Molecular Biology Grade	120 µl	1 ml	-20°C after opening

Introduction

The Monarch HMW DNA Extraction Kit for Tissue provides a rapid and reliable process for extracting high molecular weight (HMW), intact genomic DNA from various tissues and bacteria, as well as other sample types including yeast, insect, and amphibian. The optimized extraction protocol for tissue utilizes pestle homogenization and proteinase K digestion with agitation for sample lysis, followed by a protein removal step and precipitation of the extracted DNA onto the surface of large glass beads. A slightly modified extraction protocol for bacteria utilizes lysozyme for the efficient lysis of the bacterial cell wall prior to proteinase K digestion. DNA size ranges from 50 – ≥ 500 kb for the standard protocols and can be adjusted to produce longer DNA into the Mb range for soft organ tissues and bacteria. Purified DNA is recovered in high yield with excellent purity, including nearly complete removal of RNA. For tissue and bacteria, the processing time is ~90 minutes. Purity ratios for tissue and bacteria are typically 1.8-1.9 (A_{260}/A_{280}) and 2.1-2.5 (A_{260}/A_{230}). Purified HMW gDNA is suitable for a variety of downstream applications including long-read sequencing (Oxford Nanopore Technologies® and Pacific Biosciences®), optical mapping (Bionano Genomics®), and linked-read genome assembly (10X Genomics®).

Figure 1: The Monarch HMW DNA Extraction Kit for Tissue efficiently purifies high-quality, HMW DNA from a variety of sample types.



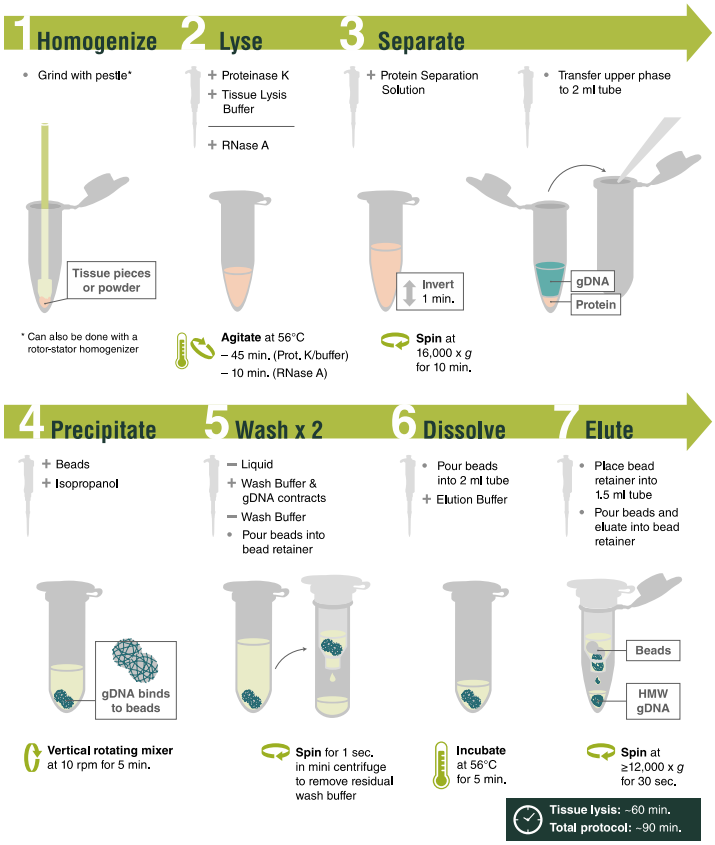
Input amount (mg)	10	10	20	20	$2x10^9$	$2x10^8$	3	$4x10^8$	15
Yield (µg)	20.8	20.2	16.9	8.9	14.5	4.2	5.2	12.9	6.7
260/280	1.86	1.86	1.86	1.85	1.86	1.87	1.85	1.90	1.84
260/230	2.41	2.26	2.45	2.28	2.06	1.95	2.33	2.01	2.53

HMW genomic DNA extracted with the Monarch HMW DNA Extraction Kit for Tissue. 10 mg frozen rat kidney, 10 mg fresh mouse liver, 20 mg frozen mouse brain, 20 mg fresh mouse muscle, 2 x 10^9 frozen E. coli cells, 2 x 10^8 frozen B. cereus cells, 4 mg fresh X. laevis, 3.8 x 10^8 fresh S. cerevisiae cells and 15 mg frozen A. aegypti were used as inputs for preps. Preps were performed according to the kit instructions with sample agitation at 2000 rpm. A modified workflow was used to process S. cerevisiae preps. 500 ng of DNA from each sample prep was resolved by PFGE (1% agarose gel, 6 V/cm, 13°C for 20 hours, switch times ramped from 0.5-94 seconds on a Bio-Rad® CHEF-DR® III System). Yield and purity ratios of the individual preps are shown in the accompanying tables. Lambda PFG Ladder (NEB #N0341) was used as a molecular weight standard.

Specifications

Input	Tissue: • Standard Input: 10 – 25 mg (10 – 20 mg for brain, 5 – 15 mg of DNA-rich or soft organ tissues, e.g., kidney, liver) • Low Input: 5 – < 10 mg (2 – < 5 mg of DNA-rich or soft organ tissues, e.g., kidney, liver) Bacteria: • Standard Input: *E. coli*: 1 x 10^9 – 5 x 10^9 cells • Low Input: *E. coli*: 5 x 10^8 – < 1 x 10^9 cells, *B. cereus*: 2 x 10^8 – 4 x 10^8 cells
Binding Mechanism	Precipitation on glass beads
Yield/Capacity	Yield depends on input amount and is not limited by the capacity of the beads. NEB has observed yields in some cases > 300 µg. Expected yields are summarized in "Choosing Input Amounts". Typical tissue protocols yield up to 60 µg of HMW DNA.
Genomic DNA Size	Using maximum agitation speed: 50 – 250 kb and ranging up to ~ 500 kb Using low agitation speed: > 50 kb up to Mb range for soft organ tissues and bacteria
RNA Content	< 2% for tissues, < 4% for bacteria
Purity	OD $_{260/280}$ typically 1.8 – 1.9; OD $_{260/230}$ typically 2.1 – 2.5
Compatible Downstream Applications	Oxford Nanopore Technologies sequencing, Pacific Biosciences sequencing, optical mapping (Bionano Genomics), linked-read genome assembly (10X Genomics)

Figure 2: Workflow for Tissue Samples



Principles of the Monarch HMW DNA Extraction Kit for Tissue

This kit employs a novel approach to the extraction of HMW DNA from tissue samples, using glass beads as a capture surface. In contrast to silica spin columns that cannot effectively release gDNA > 100 kb, the use of glass beads enables the capture and release of very high molecular weight DNA fragments. A streamlined workflow coupled with an optimized buffer system provides reproducibly high yields and highly-pure DNA extractions with exceptional convenience and speed. The method employed in this kit, as well as an overview of the principles that drive its performance, are described in detail below.

Lysis and Protein Removal

Effective and efficient lysis of biological samples is an integral step in any nucleic extraction workflow. This kit employs tissue homogenization, lysis, and a separate protein removal step to provide HMW DNA with maximal purity and yield. Tissue samples are homogenized using the included pestle or a rotor-stator homogenizer. Sample lysis employs the specialized Tissue Lysis Buffer which enables rapid and efficient lysis of multiple tissue sample types and gives maximal protection against any form of nuclease activity. Lysis is carried out with the help of Proteinase K, coupled with agitation in a thermal mixer. Agitation speeds up the lysis process and controls gDNA fragment size. After complete lysis of the sample, RNA is efficiently removed in a separate RNase A digestion step. Subsequently, residual protein is removed by adding the non-toxic Protein Separation Solution. After centrifugation and phase separation, the supernatant containing the DNA is separated from the protein phase by pipetting and is transferred to a fresh tube where it is precipitated and bound to the beads.

Tunability of DNA Fragment Length

During lysis in the thermal mixer, the chosen agitation speed determines the fragment length of the purified genomic DNA. Low-speed agitation produces the gentlest conditions and maximizes the size of the recovered gDNA (> 50 kb up to Mb range for soft organ tissues and bacteria). Maximum agitation rates during processing introduce small amounts of shear force, reducing DNA fragment sizes to 50–250 kb (ideal for ligation-based nanopore sequencing); see “Choosing Agitation Speed During Lysis”.

DNA Capture

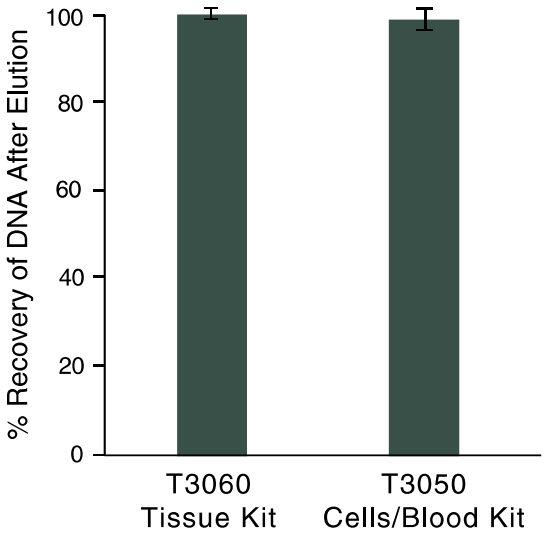
DNA extraction methods that employ magnetic beads or spin columns lead to shearing and typically only allow for the purification of DNA up to 150–200 kb. In the Monarch HMW DNA Extraction Kit, alcohol-based precipitation facilitates attachment of the genomic DNA to specialized large glass beads, which provide a smooth surface to collect precipitated DNA and prevent shearing. During the binding process, HMW gDNA attaches to the beads and subsequently wraps around the large surface of the beads during inversion. As such, the binding capacity of the bead itself becomes irrelevant. Additionally, the unique method of capture on the glass bead surface allows all captured DNA to be completely recovered in a single elution step. The capacity of the kit, which can exceed 60 µg of DNA, is limited by the working volumes of the reagents, the parameters of the protein separation and by the potential increases in viscosity that can result when using larger input amounts.

Washing and Elution

Employing glass beads as a capture surface in this workflow enables rapid washing and elution of the HMW gDNA. Following capture on the bead, the DNA is washed twice with an optimized wash buffer, and the beads (containing the DNA) are transferred to a bead retainer where a short spin ensures traces of buffer are rapidly removed.

The beads containing the bound DNA are then transferred to a 2 ml tube where the elution buffer is added. The sample is heated to 56°C for 5 minutes to release the DNA from the beads. Beads and eluate are transferred to the bead retainer, which has been inserted into a collection tube, and the eluate is separated from the glass beads with a short spin. The smooth surface of the beads allows the DNA to be eluted completely and to go into solution relatively easily; therefore, there is no need for extensive manipulation during and after elution (drying of the DNA, long incubations with elution buffer, overnight dissolving etc.). For optimal DNA solubility, avoid letting the bound DNA dry out on the beads.

Figure 1: The Monarch HMW DNA Extraction Kits enable complete recovery of input DNA.



NEB #T3050: 10 µg lambda DNA (~20 µl, NEB #N3011) was combined with Monarch gDNA Nuclei Prep Buffer to bring the total volume to 50 µl. The Cell Protocol (low input) was carried out from Step 4, omitting enzymes and enzyme incubation steps. NEB #T3060: 10 µg lambda DNA (~20 µl, NEB #N3011) was combined with Monarch HMW gDNA Tissue Lysis Buffer to a total volume of 100 µl. The Tissue Protocol (for 'very low input' samples) was carried out from Step 8, while omitting enzymes and enzyme incubation steps. DNA concentration of 100 µl eluates from each kit was measured by OD assessment. Average recovery rate and standard deviation were calculated based on 8-fold replicates.

Important Considerations for Starting Materials

Tissue Samples

HMW DNA can be isolated from fresh or frozen tissue. In general, fresh tissue samples should be processed immediately. If processing of fresh tissue samples is delayed for several hours, the quality of the isolated HMW DNA may be lower, particularly for metabolically active organ tissues. Alternatively, samples may be frozen and stored at -80°C. If working with fresh or frozen tissue pieces, ensure the tissue is cut into the smallest pieces possible.

Adequate sample storage can be carried out by one of two methods: A) Flash-frozen tissue samples can be stored as whole pieces at -80°C. B) Flash frozen tissue samples can be pulverized in liquid nitrogen using a mortar and pestle, and subsequently stored at -80°C as tissue powder. Frozen tissue powders are recommended over frozen tissue pieces, as lysis is more efficient, and handling is easier. Freshly or recently prepared frozen tissue powder samples provide better yields than those stored long term at -80°C. Working with stabilized tissue (e.g., RNAlater®) is possible, but is not the preferred option, as fragment sizes may be reduced when compared to fresh and frozen tissue samples.

- **Fresh Tissue Samples**
  - Weigh out the exact amount by transferring small tissue pieces into a reaction tube positioned on a micro balance.
  - Keep tubes cold and process as soon as possible.
  - Do not use more tissue than recommended (see "Choosing Input Amounts").

- **Frozen Tissue Powders**
  - Label and pre-cool reaction tubes on dry ice. Keep tubes containing tissue powder on dry-ice and use small pre-chilled scoops that allow for the transfer of 2 or 10 mg frozen tissue powder at a time.
  - Tare pre-chilled tube on the micro balance and transfer the appropriate amount of frozen tissue powder to tube for weighing. Do not use more tissue than recommended (see "Choosing Input Amounts"). Work quickly to prevent the tube from warming up on the balance.
  - Keep the aliquoted samples on dry ice to ensure the powder stays frozen.

- **Frozen Tissue Pieces**
  - Keep samples frozen (e.g., by storing on dry ice).
  - Use a clean, frozen cooling block or the bottom side of a frozen metal microfuge tube stand for cutting frozen tissue. Samples are most easily cut when they are processed shortly before thawing.
  - Weigh the desired amount by transferring small tissue pieces into a pre-chilled reaction tube positioned on a micro balance. Do not use more tissue than recommended (see "Choosing Input Amounts").
  - Keep tubes frozen or on ice, and process as soon as possible. In samples that have been frozen, ice crystals have destroyed cell structures and nucleases have free access to the genomic DNA. Work with the smallest possible tissue pieces to allow for efficient homogenization and rapid inactivation of nucleases by Proteinase K.

- **Stabilized Tissue Pieces**
  - Tissue samples that are stored in stabilization reagents (e.g., Monarch DNA/RNA Protection Reagent, RNAlater) can also be processed. However, working with fresh or frozen tissue without stabilization reagents is recommended for optimal purity, yield, and longer, more uniform DNA fragment size.
  - When working with samples stored in RNAlater, completely remove the reagent from the tissue sample and follow the standard protocol.
  - If working with samples stored in 1X Monarch DNA/RNA Protection Reagent, transfer the reagent from the tissue sample to a different tube, but do not discard. Homogenize the tissue sample with the supplied pestle and tube. Add Proteinase K to the reagent that was transferred to a different tube and use this Protection Reagent/PK mixture in place of the Monarch HMW gDNA Tissue Lysis Buffer supplied with the kit. Unfortunately, sample lysis may be incomplete and small tissue pieces may still be visible at the end of the lysis incubation. Adding 1/10 volume of Monarch Proteinase K Reaction Buffer, which is a component of the Monarch Total RNA Enzyme Pack (NEB #T2019L), to the 1X solution will increase lysis efficiency. Upon adding the Protein Separation Solution, the subsequent centrifugation step will not result in phase separation; instead, a pellet with small tissue particles will form. Transfer all of the supernatant to a new 2 ml tube and follow the standard protocol.

- **Special Considerations for Specific Tissue Types**

  Liver: Liver samples are rich in the polysaccharide glycogen. When isolating DNA from liver samples, glycogen may coprecipitate with DNA and affect DNA purity, resulting in variable or low A_{260}/A_{230} values. Addition of the appropriate concentration of salt (NaCl) to a liver lysate before DNA precipitation helps to remove polysaccharides. Specifically, when DNA is precipitated in the presence of 2.2 M NaCl, polysaccharides remain in solution and are discarded, typically resulting in purified DNA with A_{260}/A_{230} values > 2.0. This protocol modification is provided after Step 11, in Part 1 of the Tissue Protocol.

  Brain: Brain is a fatty tissue. During pestle homogenization, the fatty material tends to stick and may accumulate on the upper rim of the pestle. For maximal yields, this tissue material should be returned to the lysate using the tip of a p200 pipette tip. Reducing agitation time from 45 minutes to 15 minutes during lysis may improve yields from brain samples; yields may be up to 50–100% higher, particularly when working with input amounts close to the lower end of the input range. The high fat content of brain samples also affects the phase separation and typically requires longer centrifugation times to be complete. Chilling the lysed sample for 3 minutes before adding the Protein Separation Solution will facilitate the phase separation and the isolated DNA will be of higher purity. The high fat content of brain samples limits the maximum input amount to 20 mg; above that, a complete phase separation will not be achieved with the given liquid volumes.

  Muscle: Muscle is a fibrous tissue. Reducing agitation time from 45 minutes to 15 minutes during lysis will improve yields from muscle samples; yields may be up to 50–100% higher, particularly when working with input amounts close to the lower end of the input range. During lysis, insoluble protein fibers will float on the surface of the upper phase after the phase separation. For highest purity, avoid transferring these fibers. A simple way to get rid of a substantial amount of the fibers is to dip the p1000 wide-bore tip into the upper phase when starting the transfer of the upper phase. Many fibers will stick to the outside of the pipette tip and will remain stuck to the plastic material during the further transfer process.

  Mouse and Rat Tail Tips and Ear Punches: Mouse and rat tail tissues are very rigid and fibrous, making homogenization and lysis challenging. As rotor-stator homogenization is not effective for this tissue type, manual homogenization with a microtube pestle is recommended. However, even intensive grinding with a pestle will not allow for complete homogenization because of the toughness of the tail material. Before starting homogenization and lysis, tail samples should be cut into very small pieces (1-2 mm) with surgical scissors or a razor blade. Lysis at the highest agitation speed (2000 rpm) is recommended to speed up lysis. However, for isolation of the largest possible HMW DNA, agitation can be stopped or reduced to 500 rpm once the tissue pieces are fully lysed (5-10 minutes). Because of the challenges with lysis, HMW DNA isolated from tail tips tends to have a lower average length than DNA from organ tissue. Depending on the downstream application, a size selection step may be considered.

The recommended input amount for tail tips is 20 mg, although up to 25 mg (~8 mm tail tip) can be processed; expected yields are ~ 2 µg per mg. Tail tissue becomes more rigid with increasing animal age, and as such, best results are obtained with younger animals. Fresh samples will yield higher DNA quality than frozen ones, as tissues that have been previously frozen are more susceptible to nucleases during lysis.

Like tail tips, ear punches are also tough and difficult to lyse. Ear punches should be cut into very small pieces to facilitate lysis, which, in contrast to organ tissue, will take several minutes. During lysis, ear punches (and skin tissue in general) will release insoluble fibers which tend to float on the surface of the upper phase after the protein removal step; avoid transferring any of these fibers. The recommended input amount is 10 mg, and because of the fiber issue, not more than 15 mg should be used. Samples 5-10 mg should be processed as "low input" samples with reduced lysis volumes. If processing very low input amounts (2-5 mg), review the guidance in "Using Very Low Input Amounts". Expected DNA yield is ~1.5 µg per mg tissue.

Bacterial Samples (Gram-negative and Gram-positive)

In addition to tissue, the Monarch HMW DNA Extraction Kit for Tissue provides a rapid and reliable process for extracting HMW genomic DNA from bacteria. Separate protocols for processing Gram-negative and Gram-positive bacteria are provided and differ slightly in the initial lysis step. Lysozyme is required to efficiently lyse the bacterial cell wall in these tough-to-lyse samples, and for processing Gram-positive bacteria, a STET buffer is also required. Alternative lysis enzymes (e.g., lysostaphin) may be required for certain Gram-positive bacteria. Ensure that the recommended input amounts of cells are used by measuring the density of bacteria cells in liquid culture (see "Choosing Input Amounts"). Cells should be pelleted, and all culture medium removed prior to sample processing. Standard and low input protocols are provided to ensure the buffer volumes are appropriate for the sample input amount used.

Choosing Input Amounts

Recommended input amounts are provided in the accompanying table. It is strongly advised to use these recommended amounts for optimal results. Due to the unique binding process in this protocol, typically, all the DNA in the sample will bind to the glass beads effectively when standard sample input amounts are processed. Within the standard input range, all the DNA that is bound to the beads will elute completely, providing complete, or nearly complete, recovery of the DNA present in the sample. If working with low (or very low) sample inputs, the binding efficiency of system will be somewhat reduced, and the yields will be as well. Reduction of the lysis volume will improve the binding efficiency and yield in these cases. When working with low or very low amounts, follow the detailed guidance that is provided.

Tissue

The sample input range is 2–25 mg for most tissues (2–15 mg of DNA-rich/soft organ tissues (e.g., kidney, liver), 2–20 mg for brain). The upper limit for tissue input amounts is often limited by the viscosity of the lysed sample, which negatively impacts enzyme access, protein removal, precipitation onto the beads, and dissolving/resuspension of the purified DNA. In some samples, the high amounts of fibers or fatty acids can be factors that limit the input amounts. If a lower-than-recommended input amount is used, DNA recovery will be significantly reduced. Standard and low input protocols are provided to ensure the buffer volumes are appropriate and that precipitation onto the beads is efficient. If working with fatty or fibrous tissues (e.g., brain and muscle), and only very small amounts of sample are available (< 5 mg), see guidance in "Using Very Low Input Amounts".

Bacteria

The sample input range for *E. coli* is $5 \times 10^8 – 5 \times 10^9$ cells. As described for tissue samples, the upper limit for bacteria input amounts is limited by the viscosity of the lysed sample, which negatively impacts enzyme access, protein removal, precipitation onto the beads, and dissolving/resuspension of the purified DNA. If an input amount below the recommended amount is used, DNA recovery will be significantly reduced. Standard and low input protocols are provided to ensure the buffer volumes are appropriate for the sample input amount used. The sample input range for *B. cereus* using the low input protocol is $2 \times 10^8 – 4 \times 10^8$ cells.

Yeast

The recommended sample input amount for *S. cerevisiae* is 20×10^7 (20 OD) fresh, log phase cells. Using more than 20 OD cells is not recommended as higher inputs will negatively impact protein removal (i.e. phase separation) and precipitation of DNA onto capture beads. If a lower-than-recommended input amount is used, DNA recovery will be significantly reduced. The standard yeast protocol for processing 20 OD cells ensures that the buffer volumes are appropriate and DNA precipitation onto the beads is efficient.

Detailed Input Amount Guidance

The table below provides guidance on the minimum, maximum, and recommended input amounts for various sample types when using the Monarch HMW DNA Extraction Kit for Tissue. Data on yield, purity and RNA content is also provided. Samples that were successfully tested in standard ligation-based Oxford Nanopore Technologies sequencing runs are indicated. Using input amounts that exceed the

maximum will lead to challenges in solubility and viscosity, and purity may be affected. If more starting material is required, splitting the sample and performing multiple preps is recommended. RNA content was determined by HPLC analysis of nucleoside content after digestion of 1 µg of eluted nucleic acid with the Nucleoside Digestion Mix (NEB #M0649). Using input amounts below the recommended minimums will reduce yields drastically.

Table 1: Guidance on sample input amount and expected results

		MINIMUM INPUT (mg)	MAXIMUM INPUT (mg)	RECOMMENDED INPUT (mg)	YIELD (µg) FOR RECOMMENDED INPUT (YIELD PER mg)	PURITY RATIOS		RNA CONTENT	VALIDATED FOR ONT SEQUENCING?
						A260/280	A260/230		
Mammalian Tissue									
Mouse brain	Fresh	2**	20	15	12–21	1.87	2.39	ND	YES
	Frozen	2**	20	15	15–21 (1–1.5)	1.86	2.48	ND	YES
Mouse liver	Fresh (w/NaCl)	2 *	15	10	7	1.84	2.10	1.2%	YES +++
	Frozen (w/NaCl)	2 *	15	10	17–19 (1.7–1.9)	1.89	2.50	ND	YES +++
	Fresh*	2 *	15	10	20	1.84	1.52+	8.7%	YES +++
	Frozen*	2 *	15	10	27–31 (2.7–3.1)	1.89	1.93++	ND	YES +++
Mouse muscle	Fresh	2**	25	20	8–9	1.87	2.25	2.1%	YES
	Frozen	2**	25	20	12–16 (0.6–0.8)	1.87	2.30	ND	YES
Mouse kidney	Fresh	2 *	15	10	23–34	1.86	2.44	ND	YES +++
	Frozen	2 *	15	10	32–41 (3.2–4.1)	1.86	2.53	0.8%	YES +++
Mouse tail	Frozen	2**	25	20	20 (1.8–2.1)	1.86	2.43	ND	YES+++
Mouse ear punch	Fresh	2**	15	10	15–16 (1.5–1.6)	1.86	2.29	ND	YES +++
Rat kidney	Frozen	2	15	10	20–25	1.87	2.40	ND	YES
Bacteria									
E. coli (Gram-negative)	Frozen	5 x 10^8 cells	5 x 10^9 cells	1 x 10^9 cells	8–9	1.89	2.31	1.7%	YES
B. cereus (Gram-positive)	Frozen	2 x 10^8 cells	4 x 10^8 cells	2 x 10^8 cells	4–5	1.86	2.20	3.9%	YES +++
M. luteus (Gram-positive)	Frozen	ND	ND	1 x 10^8 cells	2.0	1.89	2.09	ND	ND
Amphibian									
X. laevis	Fresh	ND	ND	3–4	5	1.86	2.51	2.3%	ND
Yeast									
S. cerevisiae	Fresh	ND	ND	20 x 10^7 cells	3–6***	1.90	2.01	ND	ND +++
Insect									
A. aegypti	Frozen	ND	ND	15	6	1.84	2.53++	2.7%	ND
Nematode									
*C. elegans*****	Frozen	ND	ND	2 plates	8.2	1.91	2.5	ND	ND

ND = Not determined

* Standard protocol without recommended NaCl treatment.

** If working with input amounts < 5 mg, refer to the product manual for guidance on reducing buffer volumes.

*** Total nucleic acid yields are 4-10 µg and 6-12 µg for haploid and diploid strains, respectively. Though an RNase A step is included, RNA is co-purified. Yields may vary depending on the strain.

**** Rotor-stator homogenization is recommended.

\+ Measured with Nanodrop One; systems that differentiate turbidity in the content profiling will give higher values.

++ Measured with Unchained Labs Lunatic (formerly Trinean DropSense16); devices without content profiling that differentiates turbidity may give lower values.

+++ Size selection is recommended.

Using Very Low Input Amounts

In some cases, inputs below the range provided can be successfully processed using this kit if the volume of lysate, reagents and buffers are reduced, as described below. For brain and muscle samples, input amounts of 2–5 mg have been successfully processed. When working with samples in this input range, stop agitation after 15 minutes during the 45-minute lysis for maximum yield.

- Part 1, Step 1: use 100 µl Tissue Lysis Buffer and 10 µl Proteinase K
- Part 1, Step 6: use 5 µl RNase A
- Part 1, Step 8: use 55 µl Protein Separation Solution
- Part 2, Step 2: use 90 µl isopropanol

Homogenization Techniques

Fresh and frozen tissue (pieces and powders) can be homogenized by pestle or rotor-stator homogenizer (e.g., TissueRuptor® II). A disposable pestle and pestle tube are provided in the kit. Importantly, the homogenization method, as well as the use of fresh or frozen tissue, influences the size of the HMW DNA that is extracted. Pestle homogenization of fresh or frozen tissue typically produces gDNA 50 kb to ≥ 500 kb. Similarly, rotor-stator homogenization of fresh tissue typically produces gDNA 50 kb to ≥ 500 kb. Rotor-stator homogenization of frozen samples generates shorter fragments, 50–250 kb gDNA, which may improve ligation-based nanopore sequencing results.

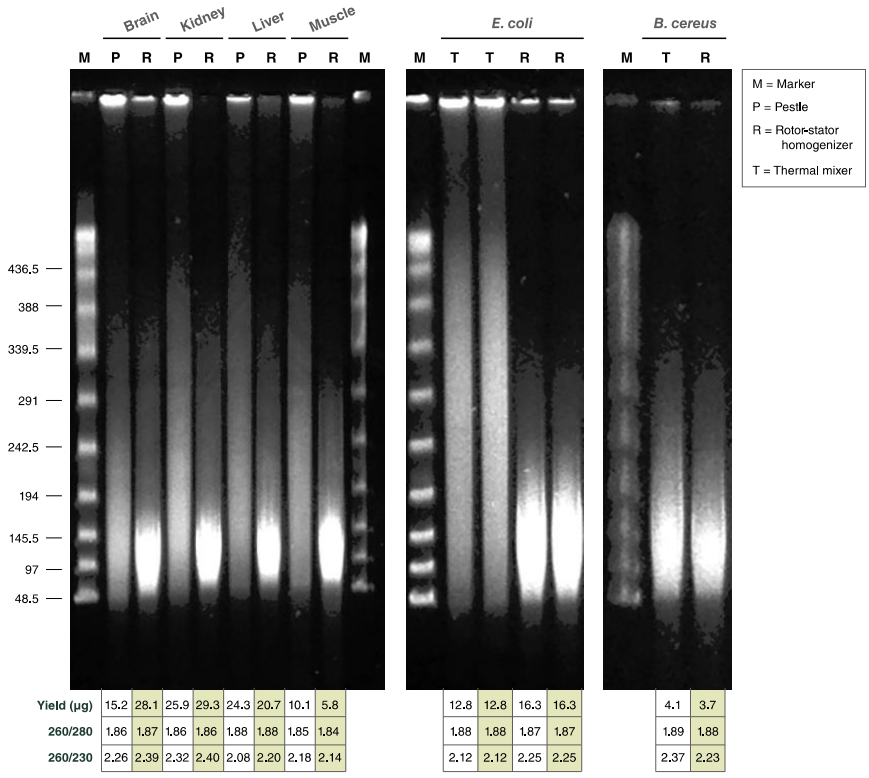
Pestle Homogenization

Tissue samples can be quickly and conveniently homogenized using a disposable pestle and pestle tube included in the kit. The pestle is used to grind the tissue sample within the pestle tube. As homogenization initiates endogenous nuclease activity, the homogenized tissue is immediately resuspended in Tissue Lysis Buffer containing Proteinase K and incubated in a thermal mixer, ensuring that nucleases can rapidly be degraded, resulting in HMW, intact genomic DNA.

Rotor-Stator Homogenization

Tissue samples may be homogenized using a rotor-stator homogenizer (e.g., TissueRuptor II). The tissue sample is placed in a 2 ml tube (not provided) with Tissue Lysis Buffer containing Proteinase K. Using the lowest setting on the rotor-stator homogenizer, the sample is homogenized in 5–20 seconds. The homogenized sample is then immediately incubated in a thermal mixer to ensure that nucleases are rapidly degraded.

Figure 3: Pestle and rotor-stator homogenization techniques produce different fragment lengths.

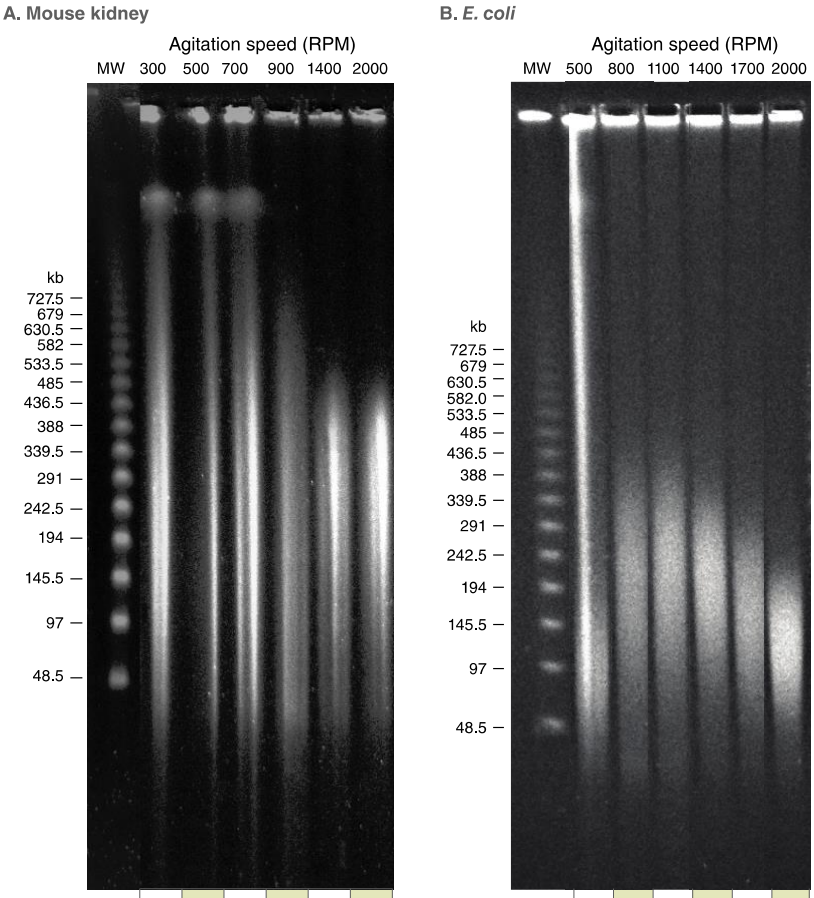


HMW genomic DNA was purified from soft organ tissues (mouse kidney, mouse liver, 10 mg), fatty (mouse brain, 20 mg), and fibrous (mouse muscle, 20 mg) tissues along with Gram – and Gram + bacteria (E. coli, 2 x 10^8 cells and B.cereus, 2 x 10^9 cells) were purified using the Monarch HMW DNA Extraction Kit for Tissue with various homogenization methods: pestle (P), rotor-stator homogenizer TissueRuptor II) I, or thermal mixer (T). 300 ng DNA of each sample was resolved on a Pippin Pulse™ gel (Sage Science) with the program for 5–430 kb. Vigorous shearing induced by the rotor-stator homogenizer reduced fragment length compared to pestle homogenization + agitation in a thermal mixer.

Choosing Agitation Speed During Lysis

The Monarch HMW DNA Extraction Kit enables users to tune the size of extracted DNA by varying the agitation speed used during lysis. Higher agitation speeds reduce overall size, as increasing the agitation speed fragments the DNA. In general, fresh samples have more intact DNA and will require higher agitation rates to shear. At 2000 rpm, the maximal fragment length obtained will be ~250–300 kb, with the majority of DNA between 100–200 kb. For the standard ligation-based Oxford Nanopore Technologies (ONT) sequencing protocol, agitation at 2000 rpm is recommended. At 300–700 rpm or with no shaking, maximal fragment length, in the Mb range (UHMW DNA), will be obtained. These samples will be highly viscous and difficult to process.

Figure 4: Use of varying agitation speeds during lysis produces tunable fragment length of extracted HMW genomic DNA from soft organ tissues and bacteria.



	300	500	700	900	1400	2000
Yield (µg)	15.6	18.1	17.3	14.9	20.0	24.2
260/280	1.88	1.88	1.86	1.86	1.85	1.87
260/230	2.20	2.33	2.34	2.33	2.37	2.43
DIN	9.6	9.8	9.8	9.4	9.8	9.8

	500	800	1100	1400	1700	2000
Yield (µg)	6.2	5.9	5.5	8.1	7.8	9.3
260/280	1.88	1.88	1.88	1.87	1.89	1.89
260/230	2.59	2.32	2.21	2.21	2.59	2.29

HMW genomic DNA from mouse kidney (10 mg, Figure 3A) and E. coli NEB10 beta (1 x 10^9 cells, Figure 3B) was purified using the Monarch HMW DNA Extraction Kit for Tissue. Samples were agitated at the indicated speed during the lysis step to control the fragmentation of the DNA. 300 ng (kidney) and 500 ng (E. coli) of DNA for each sample was resolved by PFGE (settings: switch time 0.5 to 94 sec; run time 20 hours at 6 V/cm, angle 120°, temperature 13°C on the BioRad CHEF-DR III System). Lambda PFG Ladder (NEB #N0341) was used as molecular weight standard. Yield, purity ratios and DIN values (kidney) of the individual preps are shown in the accompanying tables.

Handling and Storage of HMW DNA

High molecular weight genomic DNA is viscous, making handling and manipulation challenging. Gentle handling as well as the use of wide-bore pipette tips will help maintain the integrity of the DNA molecules. Additionally, when working with HMW DNA, tubes and pipette tips specifically designated as DNA low binding should be used. If an agitation speed of 2000 rpm was used during lysis, DNA will be in the range of 50–250 kb and the solution will be less viscous and easier to handle. Following homogenization, these samples do not require the use of wide-bore pipette tips and can even be vortexed briefly without affecting DNA integrity. When working with DNA purified using low agitation speeds during lysis (UHMW DNA) or with DNA purified from large input amounts, DNA will be especially viscous and difficult to handle. These samples should always be pipetted using appropriate wide-bore pipette tips. Pipetting these samples is challenging, as sticky threads are formed between the pipette tip and the sample as aliquots are taken. Briefly pressing the tip on the bottom of the tube can help to break these threads. Additionally, heating the sample to 37°C or 56°C for a short period of time will reduce viscosity and facilitate pipetting.

The elution buffer provided with the kit (10 mM Tris-Cl pH 9.0, 0.5 mM EDTA) is developed as a long-term storage buffer. The combination of EDTA and high pH provides optimal protection against nucleases. If the sample will be actively used, it is recommended to store HMW samples at 4°C; for long term storage, store at -20°C. Avoid repeated freeze thawing and always use low bind tubes to prevent DNA from binding to the tube walls.

Protocol: High Molecular Weight DNA Extraction from Tissue

MATERIALS REQUIRED BUT NOT SUPPLIED

- Thermal mixer containing a 1.5 ml tube block (optional: 2 ml tube block for elution)
- Isopropanol, 550 µl per sample (Low Input: 275 µl/sample)
- Ethanol (≥ 95%)
- 1.5 ml DNase-free, low DNA binding microfuge tubes (e.g., Eppendorf® DNA LoBind®, #0030108051) for elution and storage (1 per sample); it is especially important to use low DNA binding tubes if working with UHMW DNA
- Recommended: vertical rotating mixer (e.g., Thermo Scientific® HulaMixer® Sample Mixer)
- Wide-bore pipette tips (e.g., Aerosol Filter Wide Orifice Pipette Tips, VWR)
- Microcentrifuge
- Optional: rotor-stator homogenizer (e.g., TissueRuptor II), and compatible 2 ml tubes
- Optional: 5 M NaCl for liver samples

IMPORTANT NOTES BEFORE YOU BEGIN

- Review the complete protocol before beginning
- Add ethanol (≥ 95%) to the gDNA Wash Buffer as indicated on the bottle label
- Preheat thermal mixer with 1.5 ml block to 56°C

STARTING MATERIAL NOTES

- Use the table below to determine the designation of your sample type, which will determine various volumes in the protocol. If processing liver samples, follow specific guidance provided after Step 11 in Part 1.

PROTOCOL DESIGNATION	TISSUE AMOUNT
Standard Input	10 – 25 mg for most tissues 5 – 15 mg of DNA-rich or soft organ tissues (e.g., kidney, liver), 10 – 20 mg for brain
Low Input	5 – < 10 mg* or 2 – < 5 mg of DNA-rich or soft organ tissues (e.g., kidney, liver) *For brain and muscle samples, lower inputs can be successfully processed with protocol modifications; see guidance in “Using Very Low Input Amounts”.

- Fresh Tissue
  - Ensure tissues are cut into the smallest possible pieces
  - Pestle homogenization typically produces gDNA 50 kb to ≥ 500 kb
  - No performance difference has been observed using a rotor stator homogenizer

- Frozen Tissue
  - Tissue powders are recommended over tissue pieces, as lysis is more efficient, and handling is easier
  - Freshly or recently prepared frozen tissue powder samples provide better yields than those stored long term at -80°C
  - If working with a powder is not possible, ensure frozen tissue is cut into the smallest possible pieces
  - Rotor-stator homogenization has been shown to improve ligation-based nanopore sequencing by generating shorter gDNA fragments (50–250 kb)
  - Samples homogenized using the pestle may benefit from needle shearing after elution. See guidance in "Needle Shearing".

Genomic DNA Purification Consists of Two Stages:

PART 1: Tissue Lysis

PART 2: HMW gDNA Binding and Elution

PART 1 TISSUE LYSIS

1. **Prepare a master mix of HMW gDNA Tissue Lysis Buffer and Proteinase K according to the table below and the number of samples that will be processed.** There is no need to make excess master mix.

PROTOCOL DESIGNATION	VOLUME OF HMW gDNA TISSUE LYSIS BUFFER PER SAMPLE (µl)	VOLUME OF PROTEINASE K PER SAMPLE (µl)
Standard Input	600	20
Low Input	300	10

2. **Transfer the desired amount of tissue to a Monarch Pestle Tube (or, if using a rotor-stator homogenizer, to a compatible 2 ml tube, not included). Place on ice (fresh samples) or dry ice (frozen samples).**

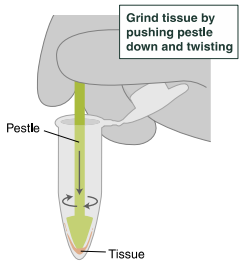
3. **Briefly spin the sample (2–3 seconds) in a benchtop minicentrifuge to collect all tissue material at the bottom of the tube.** For frozen samples, thaw samples one at a time at room temperature, spin down, and proceed quickly to homogenization. Then, repeat for any remaining samples. If working with frozen tissue powder and some of the powder is stuck to the tube, let the sample thaw briefly before spinning and proceed immediately to homogenization.

4. **Homogenization can be carried out using one of two methods: using a pestle or rotor-stator homogenizer.** If using a rotor stator homogenizer, the sample must be in a 2 ml tube (not provided). If working with multiple samples, each sample should be taken quickly through all sub-steps and placed in the thermal mixer before processing the next sample. Homogenization initiates endogenous nuclease activity, making it essential to take each sample through to incubation before starting the next sample.

Homogenization Using Pestle

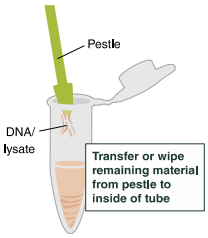
⚠ If working with multiple samples, perform all sub-steps for one sample and then repeat with the next sample.

a. **Use the pestle to grind the sample within the pestle tube; leave the pestle in the tube.**



b. **Using a wide-bore pipette tip, add 600 µl (Low Input: 300 µl) of the lysis master mix to the sample.** Do not dispose of this tip yet, as it will be used to mix the sample.

c. **Ensure there is no tissue material remaining on the pestle, then discard the pestle.** If visible DNA threads or tissue material stick to the pestle, transfer it carefully into the tube by wiping the pestle tip along the tube rim. When working with brain tissue, some tissue material may accumulate at the rim of the pestle tip. Remove this material using the tip of a regular 200 µl pipette tip and transfer it back into the tube.



d. **Using the wide-bore tip, pipette up and down a few times to homogenize the tissue lysate and ensure rapid, complete lysis.** Discard the pipette tip.

e. **Begin incubation in the thermal mixer (Step 5), and repeat steps a-e with any remaining samples.**

Homogenization Using Rotor-Stator Homogenizer

If working with multiple samples, perform all sub-steps for one sample and then repeat with the next sample.

a. **Add 600 µl (Low Input: 300 µl) of the lysis master mix to the sample**. If sample is not in a 2 ml tube (not provided), transfer at this step.

b. **Ensure the tissue pieces can move freely by flicking or vortexing briefly.**

c. **Insert the tip of the homogenizer probe into the sample and turn on to the lowest setting. Homogenize until tissue pieces are no longer visible.** Suggested homogenization time is 5–20 seconds; stop when foam begins to form in the lysate. Additional homogenization may be required to reach optimal gDNA size for ONT sequencing (50–250 kb). gDNA size can be verified by pulse field gel electrophoresis or FEMTO Pulse®. Rotor-stator homogenizers may run at higher speeds after extended use; reduce homogenization time if necessary.

d. **Transfer sample to a 1.5 ml Pestle Tube and begin incubation in the thermal mixer (Step 5). Exchange or clean the rotor stator probe as recommended by the manufacturer and repeat steps a-e with remaining samples.**

5. **Incubate samples in a thermal mixer at 56°C for a minimum of 45 minutes with agitation at the desired speed. When lysis is complete, samples will turn from turbid to clear (or mostly clear, depending on the tissue type).** The speed of the thermal mixer influences fragment length and lysis; higher agitation speeds reduce DNA size and sample lysis time. For most applications, including the standard ligation-based Oxford Nanopore Technologies (ONT®) sequencing protocols, maximum agitation speed (1400-2000 rpm) is recommended.
    - Maximum agitation speed (1400–2000 rpm) will produce DNA fragments predominantly 50–250 kb and ranging up to ~500 kb. Under these agitation conditions, tissue samples will be rapidly digested and are easy to process.
    - For maximum DNA length (UHMW DNA, up to Mb range for soft organ tissues), agitate at 500–700 rpm. During incubation, mix samples every 10–15 minutes (more frequently for DNA-rich samples) by inverting a few times to ensure complete lysis. With low agitation speeds, tissue samples are digested very slowly, and the isolated HMW gDNA is significantly “tangled”, reducing the efficiency of protein removal in later steps. This DNA is also difficult to dissolve during elution and can result in visible DNA aggregates.
    - For highest yields, particularly when working with low input samples or samples with relatively low DNA content (e.g., brain and muscle), stop agitation after 15 minutes and continue the remaining incubation time without agitation.

6. **Add 10 µl (Low Input: 5 µl) RNase A and mix by inverting 5–10 times. Incubate for 10 minutes at 56°C at the same agitation speed used in Step 5.** If working with brain tissue: after RNase A digestion, chill the lysate on ice for 3 minutes before adding the Protein Separation Solution to ensure efficient phase separation.

7. **Change the heat block in the thermal mixer to accommodate a 2 ml tube, and preheat the block to 56°C.** If a 2 ml heat block is not available, continue working with the 1.5 ml block.

8. **Add 300 µl (Low Input: 150 µl) Protein Separation Solution and mix by inverting for 1 minute**. Alternatively, a vertical rotating mixer at 20 rpm can be used.

9. **Centrifuge for 10 minutes at 16,000 x *g*. If working with multiple samples, during centrifugation, prepare the plastics for Part 2, as indicated in the following step**. The sample will separate into a large, clear phase (DNA) and a smaller (often brown) protein phase, usually on the bottom of the tube. For some tissues, the protein phase may be yellow or clear. Additional centrifugation time (10–20 minutes) may be required for complete phase separation, particularly when low agitation speeds were used.

10. **If working with multiple samples, prepare and label the plastics for the upcoming steps**. Each sample will require:
    - 1 Monarch Spin Collection Tube (no need to label)
    - 1 Monarch Bead Retainer inserted into the collection tube; this will be used to remove the wash buffer from the gDNA bound to the beads.
    - 2 Monarch 2 ml Tubes; one for phase separation and one for elution.
    - 1 1.5 ml microfuge tube (DNA low bind recommended, not provided); this will be used to collect the eluate.

11. **Using a 1000 µl (Low Input: 200 µl) wide-bore pipette tip, transfer the upper phase containing the DNA (large, clear phase) to a labeled Monarch 2 ml Tube.** A substantial fraction of HMW DNA will be located at the interface between the clear upper phase and the protein phase; highest yields will be achieved by transferring as much of the upper phase as possible. Using a 200 µl wide-bore pipette tip to transfer the final volume of upper phase is recommended for maximum yield. Avoid transferring material from the protein layer, although a small amount (1–2 µl) will not be detrimental. If a small amount of the protein phase enters the pipette tip, gently push it back into the tube. If a lower protein phase is not visible, leave ~30 µl behind to ensure protein is not carried over. Typically, the transferred volume will be ~ 800 µl (Low Input: ~400 µl). If the volume of the sample is < 700 µl (Low Input: < 350 µl), adjust the volume of isopropanol used in Step 2 of Part 2: HMW gDNA Binding and Elution to 0.7 volumes.

    ⚠ If processing liver samples, see guidance below in "Processing Liver Samples".

Processing Liver Samples

Copurification of polysaccharides (i.e., glycogen) often results in variable or low A_{260}/A_{230} values. Addition of NaCl to the sample before DNA capture helps to remove polysaccharides, resulting in A_{260}/A_{230} values > 2.0. The following protocol modifications are recommended:

- Following Step 11 of Part 1 (phase separation): To 650 µl of the upper phase (Low Input: use all, ~400 µl), add 500 µl (Low Input: 315 µl) 5 M NaCl to reach a final concentration of 2.2 M NaCl. Pipette with a wide-bore tip or gently invert to mix.
- At Step 2 of Part 2, add 800 µl (Low Input: 500 µl) isopropanol (0.7 volumes of isopropanol per volume of upper phase +NaCl)

PART 2 HMW gDNA BINDING AND ELUTION

1. **Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube.**

2. **Add 550 µl (Low Input: 275 µl) isopropanol, close the cap, and mix on a vertical rotating mixer at 10 rpm for 5 minutes to attach DNA to the beads.**

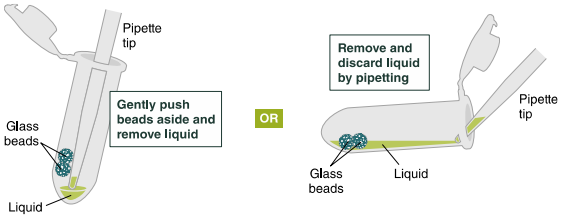
   If a vertical rotating mixer is not available, invert slowly and gently by hand 25–30 times. A manual inversion is complete when the tube returns to the upright position. Slow inversion is critical for the DNA to bind to the beads; each full inversion should take ~5–6 seconds. If necessary, flick the tube to release any beads that stick to the bottom of the tube.

   After a 2–3 inversions, the solution becomes more viscous and the DNA will wrap loosely around the beads. During the following inversions, precipitation of gDNA may be visible, especially with larger input samples. The DNA complex will often contain small air

bubbles. With increasing number of inversions, the DNA will completely wrap around the beads, often causing the beads to stick together. DNA binding to the beads should be complete after 25–30 inversions, and the solution should no longer be viscous. Additional inversions may be necessary for larger input samples.

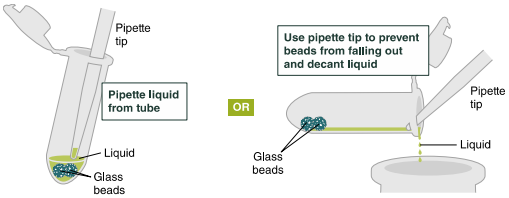
3. **Remove and discard liquid by pipetting. Avoid removing any of the gDNA wrapped around the glass beads. For optimal DNA solubility, avoid letting the bound DNA dry out on the beads during this and the following steps; add the next buffer quickly**. There are two suggested options for carrying out this step:

    - Keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.
    - Angle tube so that beads remain at the bottom, and liquid reaches toward tube opening. Pipette from the liquid surface and continue to angle as liquid is removed (tube will be almost horizontal at the end).

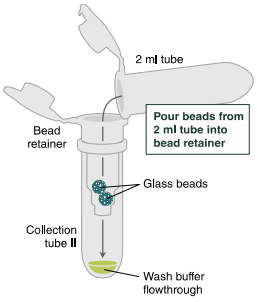


4. **Add 500 µl gDNA Wash Buffer, close the cap, and mix by inverting the tube 2–3 times. Remove the wash buffer as described in Step 3.** The loose gDNA complex will condense around the beads more tightly.

5. **Repeat the wash in Step 4 and remove the gDNA Wash Buffer by pipetting.** Alternatively, the buffer can be removed by decanting: position a pipette tip at the top of the angled tube to prevent the beads from falling out. It is not necessary to remove all the gDNA Wash Buffer at this point.

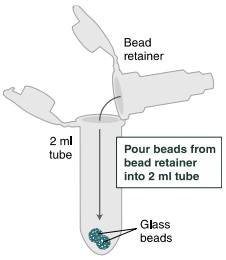


6. **Place a labeled Bead Retainer into a Monarch Spin Collection Tube. Pour the beads into the bead retainer and close the cap. Discard the used Monarch 2 ml Tube.** When working with multiple samples, be sure to close the cap of the bead retainer after each transfer of beads.

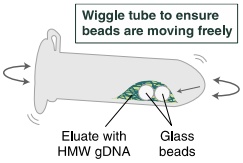


7. **Pulse spin (≤ 1 second) the sample in a benchtop minicentrifuge to remove any residual wash buffer from the beads.**

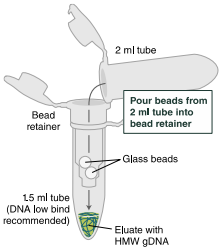
8. **Separate the Bead Retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube, and insert the used bead retainer into the labeled 1.5 ml microfuge tube for later use during elution. Discard the used collection tube.**



9. **Immediately add 100 µl Elution Buffer II onto the glass beads and incubate for a minimum of 5 minutes at 56°C in a thermal mixer with agitation at the lowest speed (300 rpm). Halfway through the incubation, ensure the beads are not stuck to the bottom of the tube by tilting the tube almost horizontally and gently shaking.** This ensures that the beads can move freely, allowing for optimal release of the DNA from the beads. It also ensures that the lower bead does not stick to the bottom of the tube during the following transfer step. Elution volume can be reduced to as low as 50 µl without affecting recovery. However, if using < 100 µl, the gentle shaking of the sample should be done several times during the incubation to ensure complete wetting of the beads.



10. **Ensure the Bead Retainer is inserted into the 1.5 ml microfuge tube (DNA low bind, not included). Pour the eluate and the glass beads into the Bead Retainer and close the cap.** When working with more than 1 sample, it is important to close the cap after each transfer of beads. Typically, all the eluate flows into the bead retainer upon pouring. If any volume remains in the 2 ml tube, spin briefly and transfer.



11. **Centrifuge for 30 seconds at 12,000 x *g* to separate the eluate from the glass beads. Discard the beads and retainer.**

12. **Pipette eluate up and down 5–10 times with a wide-bore pipette tip and ensure any visible DNA aggregates are dispersed. Before analysis or downstream use, HMW DNA must be homogeneously dissolved.** After pipetting, incubate at 37°C for 30-60 minutes, overnight at room temperature, or for > 24 hours at 4°C. Pipette up and down 5-10 times again before analyzing or using the HMW DNA. Samples processed using low agitation speeds during lysis will require additional time to fully dissolve. See additional guidance in “Homogenization of HMW DNA”. Samples can be stored at 4°C for short term use (weeks), or at -20°C for long term storage. The elution buffer (10 mM Tris, pH 9.0, 0.5 mM EDTA) is formulated for long term storage of gDNA.

Protocol: High Molecular Weight DNA Extraction from Bacteria

MATERIALS REQUIRED BUT NOT SUPPLIED

- Microcentrifuge
- Thermal mixer containing a 1.5 ml tube block (optional: 2 ml tube block for elution)
- Recommended: vertical rotating mixer (e.g., Thermo Scientific HulaMixer Sample Mixer)
- Ethanol (≥ 95%)
- Cold PBS, 300 µl per sample (Low Input: 150 µl per sample). Alternatively, TE or Tris buffer can be used.
- Isopropanol, 550 µl per sample (Low Input: 275 µl per sample)
- 1.5 ml DNase-free, low DNA binding microfuge tubes (e.g., Eppendorf DNA LoBind, #0030108051) for elution and storage (1 per sample); it is especially important to use low DNA binding tubes if working with UHMW DNA, which tends to bind to plastic surfaces.
- For gram-negative bacteria: Lysozyme (25 mg/ml, 10 µl per sample)
- For gram-positive bacteria: STET Buffer (Current Protocols in Molecular Biology) containing Lysozyme (10 mg/ml) can be an effective lysis agent (150 µl or 300 µl per sample).
- Additional lysis agents may be required (e.g., lysostaphin).

IMPORTANT NOTES BEFORE YOU BEGIN

- Review the complete protocol before beginning.
- Preheat thermal mixer with 1.5 ml block to 37°C, (if available, preheat another to 56°C)
- Add ethanol (≥ 95%) to the gDNA Wash Buffer as indicated on the bottle label.
- Rotor-stator homogenization may be used to obtain shorter gDNA fragments (50–250 kb), which often results in better ligation-based nanopore sequencing results.

STARTING MATERIAL NOTES

The volumes indicated in the protocol vary depending on input amounts. Refer to the following designations throughout the protocol to determine the appropriate volumes.

PROTOCOL	NUMBER OF BACTERIAL CELLS*
Standard Input	*E. coli*: $1 \times 10^9 – 5 \times 10^9$
Low Input	*E. coli*: $5 \times 10^8 – < 1 \times 10^9$ *B. cereus*: $2 \times 10^8 – 4 \times 10^8$

* Optimal input amounts for other bacteria may vary depending on the strain, genome size, and growth conditions.
  - *E. coli* typically produces HMW gDNA in the range of 50 kb – ≥ 500 kb
  - *B. cereus* typically produces HMW gDNA in the range of 50 kb – ≥ 350 kb

HMW gDNA Purification Consists of Two Stages:

PART 1: Bacterial Lysis

PART 2: HMW gDNA Binding and Elution

PART 1 BACTERIAL LYSIS

1. **Pellet bacterial cells in a Monarch Pestle Tube by centrifugation at maximum speed (> 12,000 x *g*) for 1 minute.** If using a rotor-stator homogenizer, use a compatible 2 ml tube, not included.

2. **Gram-negative and gram-positive bacteria are processed differently for the initial lysis steps.** The use of bead beating is not recommended as it will result in a significant reduction of gDNA size.

   ❑ **Gram-negative Bacteria**

   a. **Resuspend pellet in 300 µl (Low Input: 150 µl) cold PBS.** Cold TE or Tris buffer may be used in place of PBS if preferred.

   b. **Add 10 µl Lysozyme (25 mg/ml, not provided) and mix by vortexing briefly.**

   c. **Add 300 µl (Low Input: 150 µl) HMW gDNA Tissue Lysis Buffer to the sample and mix by inverting 5-10 times.**

   d. **Incubate at 37°C in a thermal mixer with agitation at the desired speed. The speed of the thermal mixer influences fragment length and lysis time. For most applications, maximum agitation speed (1400–2000 rpm) is recommended.** For maximum gDNA size, agitate at 500 rpm. Incubation is complete when lysate turns clear, which is approximately 3–5 minutes for *E. coli*. At 500 rpm, lysis may take longer.

   ❑ **Gram-positive Bacteria**

   a. **Resuspend pellet in 300 µl (Low Input: 150 µl) of an appropriate lysis buffer containing a lytic enzyme and mix by vortexing briefly.** STET buffer with freshly added lysozyme (10 mg/ml) works well for some *Bacillus* species.

   b. **Incubate at 37°C for 30 minutes (no agitation).**

   c. **Add 300 µl (Low Input: 150 µl) HMW gDNA Tissue Lysis Buffer to the sample and mix by inverting 5–10 times.**

3. **If working with a single thermal mixer, increase the temperature to 56°C.** Following lysozyme treatment at 37°C, increase the temperature of the block in the thermal mixer to 56°C.

4. **Add 20 µl (Low Input: 10 µl) of Proteinase K and mix by inverting 10–20 times.**

5. **Homogenization can be carried out using one of two methods, depending on the desired gDNA size: in a thermal mixer or with a rotor-stator homogenizer.** If using a rotor stator homogenizer, the sample must be in a 2 ml tube (not provided).

   ❑ **Thermal Mixer (for gDNA 50 kb up to ≥ 500 k**

   3. a. **Incubate at 56°C for 30 minutes in a thermal mixer at the desired speed**. The speed of the thermal mixer influences fragment length and lysis; higher agitation speeds reduce DNA size and sample lysis time. For most applications, including the standard ligation-based Oxford Nanopore Technologies (ONT) sequencing protocols, maximum agitation speed (1400–2000 rpm) is recommended to produce DNA fragments predominantly 50–250 kb.

      To achieve maximum gDNA size, up to the Mb range, use a low agitation speed. Agitation at speeds < 500 rpm is not recommended as gDNA will be significantly tangled, which reduces the efficiency of protein removal in later steps. This tangled DNA is also difficult to dissolve during lysis and elution and can result in visible DNA aggregates.

   ❑ **Rotor-stator Homogenizer (for gDNA 50–250 kb)**

   a. **Within a 2 ml tube, insert the tip of the homogenizer probe and turn on to the lowest setting. Homogenize 5–15 seconds; stop when foam begins to form in the lysate.** Additional homogenization may be required to reach optimal gDNA size. gDNA size can be verified by pulse field gel electrophoresis or FEMTO Pulse. Rotor-stator homogenizers may run at higher speeds after extended use; reduce homogenization time if necessary.

   b. **Transfer to a 1.5 ml Pestle Tube. Incubate at 56°C for a minimum of 30 minutes in a thermal mixer at maximum speed (1400–2000 rpm).**

6. **Add 10 µl (Low Input: 5 µl) of RNase A and mix by inverting 5–10 times. Incubate for 10 minutes at 56°C with agitation in a thermal mixer at the speed used in Step 5.**

7. **Change the heat block in the thermal mixer to accommodate a 2 ml tube, and preheat the block to 56°C.** If a 2 ml tube block is not available, continue working with the 1.5 ml block.

8. **Add 300 µl (Low Input: 150 µl) of Protein Separation Solution. Mix by inverting for 1 minute.** Alternatively, a vertical rotating mixer at 20 rpm can be used.

9. **Centrifuge for 10 minutes at 16,000 x *g*. If working with multiple samples, during centrifugation, prepare the plastics for Part 2, as indicated in the following step.** The sample will separate into a large, clear upper phase (DNA) and a lower, clear phase (protein, usually on the bottom of the tube, but occasionally floating). There may also be a white precipitate at the bottom of the tube. Additional centrifugation time (10-20 minutes) may be required for complete phase separation, particularly when low agitation speeds were used.

10. **If working with multiple samples, prepare and label the plastics for the upcoming steps. Each sample will require:**
    - Monarch Spin Collection Tube (no need to label)
    - 1 Monarch Bead Retainer inserted into the collection tube; this will be used to remove the wash buffer from the gDNA bound to the beads.
    - 2 Monarch 2 ml Tubes; one for phase separation and one for elution.
    - 1 1.5 ml microfuge tube (DNA low bind recommended, not provided); this will be used to collect the eluate.

11. **Using a 1000 µl (Low Input: 200 µl) wide-bore pipette tip, transfer the upper phase containing the DNA (large, clear phase) to a labeled Monarch 2 ml Tube.** A substantial fraction of HMW DNA will be located at the interface between the clear upper phase and the protein phase; highest yields will be achieved by transferring as much of the upper phase as possible. Using a 200 µl wide-bore pipette tip to transfer the final volume of the upper phase is recommended for maximum yield. Avoid transferring material from the protein layer, though a small amount (1–2 µl) will not be detrimental. If protein enters the pipette tip, gently push it back into the tube. If a lower protein phase is not visible, leave ~30 µl behind to ensure protein is not carried over. Typically, the transferred volume will be ~ 800 µl (Low Input: ~400 µl). If the volume of the sample is < 700 µl (Low Input: < 350 µl), adjust the volume of isopropanol used in Step 2 of Part 2: HMW gDNA Binding and Elution to 0.7 volumes.

PART 2 HMW gDNA BINDING AND ELUTION

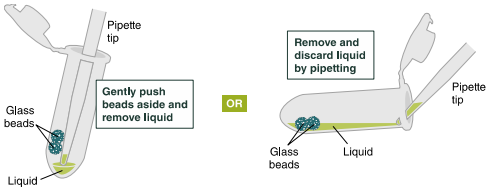
1. **Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube.**

2. **Add 550 µl (Low Input: 275 µl) isopropanol, close the cap, and mix on a vertical rotating mixer at 10 rpm for 5 minutes to attach DNA to the beads.**

   If a vertical rotating mixer is not available, invert slowly and gently by hand 30 times. A manual inversion is complete when the tube returns to the upright position. Slow inversion is critical for the DNA to bind to the beads; each full inversion should take ~5–6 seconds. If necessary, flick the tube to release any beads that stick to the bottom of the tube.

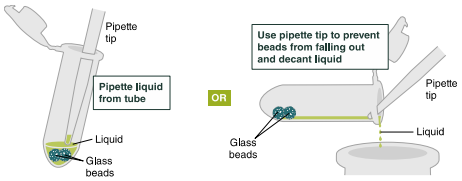
   After a 2–3 inversions, the solution becomes more viscous and the DNA will wrap loosely around the beads. During the following inversions, precipitation of gDNA may be visible, especially with larger input samples. The DNA complex will often contain small air bubbles. With increasing number of inversions, the DNA will completely wrap around the beads, often causing the beads to stick together. DNA binding to the beads should be complete after 25–30 inversions, and the solution should no longer be viscous. Additional inversions may be necessary for larger input samples.

3. **Remove and discard liquid by pipetting. Avoid removing any of the gDNA wrapped around the glass beads. For optimal DNA solubility, avoid letting the bound DNA dry out on the beads during this and the following steps; add the next buffer quickly.** There are two suggested options for carrying out this step:
    - Keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.
    - Angle tube so that beads remain at the bottom, and liquid reaches toward tube opening. Pipette from the liquid surface and continue to angle as liquid is removed (tube will be almost horizontal at the end).

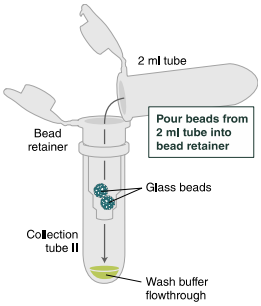


4. **Add 500 µl gDNA Wash Buffer, close the cap, and mix by inverting the tube 2–3 times. Remove the wash buffer as described in Step 3.** The loose gDNA complex will condense around the beads more tightly.

5. **Repeat the wash in Step 4 and remove the wash buffer by pipetting.** Alternatively, the buffer can be removed by decanting: position a pipette tip at the top of the angled tube to prevent the beads from falling out. It is not necessary to remove all the gDNA Wash Buffer at this point.

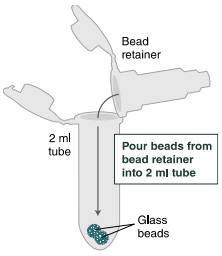


6. **Place a labeled Bead Retainer into a Monarch Spin Collection Tube. Pour the beads into the Bead Retainer and close the cap. Discard the used Monarch 2 ml Tube.** When working with multiple samples, be sure to close the cap of the Bead Retainer after each transfer of beads.

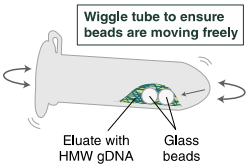


7. **Pulse spin (≤ 1 second) the sample in a benchtop minicentrifuge to remove any residual wash buffer from the beads.**

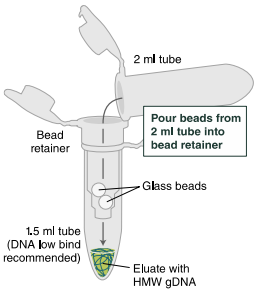
8. **Separate the Bead Retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube, and insert the used Bead Retainer into the labeled 1.5 ml microfuge tube for later use during elution. Discard the used collection tube.**



9. **Immediately add 100 µl Elution Buffer II onto the glass beads and incubate for a minimum of 5 minutes at 56°C in a thermal mixer with agitation at the lowest speed (300 rpm). Halfway through the incubation, ensure the beads are not stuck to the bottom of the tube by tilting the tube almost horizontally and gently shaking.** This ensures that the beads can move freely, allowing for optimal release of the DNA from the beads. It also ensures that the lower bead does not stick to the bottom of the tube during the following transfer step. Elution volume can be reduced to as low as 50 µl without affecting recovery. However, if using < 100 µl, the gentle shaking of the sample should be done several times during the incubation to ensure complete wetting of the beads.



10. **Ensure the Bead Retainer is inserted into the 1.5 ml microfuge tube (DNA low bind, not included). Pour the eluate and the glass beads into the Bead Retainer and close the cap.** When working with more than 1 sample, it is important to close the cap after each transfer of beads. Typically, all the eluate flows into the bead retainer upon pouring. If any volume remains in the 2 ml tube, spin briefly and transfer.



11. **Centrifuge for 30 seconds at 12,000 x *g* to separate the eluate from the glass beads. Discard the beads and retainer.**

12. **Pipette eluate up and down 5–10 times with a wide-bore pipette tip and ensure any visible DNA aggregates are dispersed. Before analysis or downstream use, HMW DNA must be homogeneously dissolved.** After pipetting, incubate at 37°C for 30-60 minutes, overnight at room temperature, or for > 24 hours at 4°C. Pipette up and down 5-10 times again before analyzing or using the HMW DNA. Samples processed using low agitation speeds during lysis will require additional time to fully dissolve. See additional guidance in "Homogenization of HMW DNA". Samples can be stored at 4°C for future use. The elution buffer (10 mM Tris, pH 9.0, 0.5 mM EDTA) is formulated for long term storage of gDNA.

Protocol: High Molecular Weight DNA Extraction from Yeast

MATERIALS REQUIRED BUT NOT SUPPLIED

- Zymolyase (5U/µl); or other yeast lytic enzyme for generating spheroplasts. This protocol was developed using Zymolyase from Zymo Research®, #E1005.
- Spheroplast Buffer: 1M sorbitol, 1X PBS pH 7.4, 0.1 M EDTA; or similar buffer to provide osmotic support in cell wall digestion step
- Cold PBS
- Cold 0.5 M NaCl
- Ethanol (≥ 95%)
- Isopropanol
- Thermal mixer containing a 1.5 ml tube block (optional: 2 ml tube block for elution)
- 1.5 ml DNase-free, low DNA binding microfuge tubes (e.g., Eppendorf DNA LoBind, #0030108051) for elution and storage (1 per sample); it is especially important to use low DNA binding tubes if working with UHMW DNA
- Recommended: vertical rotating mixer (e.g., Thermo Scientific HulaMixer Sample Mixer)
- Wide-bore pipette tips (e.g., Aerosol Filter Wide Orifice Pipette Tips, VWR)
- Microcentrifuge

IMPORTANT NOTES BEFORE YOU BEGIN

- Store Proteinase K and RNase A at -20°C upon opening
- Review the complete protocol before beginning
- Add ethanol (≥ 95%) to the gDNA Wash Buffer as indicated on the bottle label
- Preheat thermal mixer with 1.5 ml block to 30°C (if available, preheat another to 56°C)

STARTING MATERIAL AND PROTOCOL NOTES

- The following protocol has been used to isolate 3-6 µg HMW DNA from 20 x 10^7 (~20 OD) log phase *S Cerevisiae* haploid and diploid cells. While this workflow includes an RNase A digestion step, RNA is co-purified with HMW DNA to yield 4-10 ug and 6-12 ug total nucleic acids from haploid and diploid cells, respectively. Purity ratios for *S. cerevisiae* samples are typically 1.9 (A_{260}/A_{280}), reflecting residual RNA present in the sample; and 1.8-2.5 (A_{260}/A_{230}) with systems that differentiate turbidity in

the content profiling giving a higher value. For downstream applications that require removal of residual RNA, the RNase A digestion step will require further optimization (Part 2, Step 4). Specifically, users may find that the amount of RNA co-purified may be reduced by 1) extending the RNase A digestion time from 10 min to 30-60 min, and/or 2) using a larger volume of RNase A in the digestion step (e.g., 20 µl instead of 10 µl).

HMW gDNA Purification Consists of Two Stages:

PART 1: Spheroplast Preparation

PART 2: Yeast Cell Lysis

PART 3: HMW gDNA Binding and Elution

PART 1 SPHEROPLAST PREPARATION

1. **Harvest ~20 OD log-phase yeast cells by centrifugation at 5000 x *g* at 16°C for 15 minutes. Remove as much of the culture medium as possible.**

2. **Wash cells by resuspending the cell pellet in 10 ml cold PBS. Centrifuge at 5000 x *g* at 16°C for 15 minutes. Remove supernatant.** Thorough washing of cells to remove all residual culture media is important for subsequent steps, including successful DNA binding to capture beads.

3. **Repeat Step 2.**

4. **Resuspend cell pellet in 500 µl of an appropriate buffer (cold) for spheroplast preparation (i.e., a buffer that provides osmotic support).** The Spheroplast Buffer described above in Materials Required but Not Supplied, along with Zymolyase, may be used to prepare *S. cerevisiae* spheroplasts.

5. **Add yeast cell wall lytic enzyme [e.g., 10 µl Zymolyase (5 U/µl)] and pipette with a wide-bore tip to mix.**

6. **Incubate at 30°C for ~20 minutes**. Invert and gently flick tubes every 5 minutes to keep cells suspended in solution. If possible, periodically check 3-5 µl aliquots of the digestion mixture for spheroplast formation under the microscope. Proceed to the next step once ~80% of the cells are spheroplasts.

7. **If working with a single thermal mixer, increase the temperature to 56°C.** Following the sample incubation at 30°C, increase the temperature of the block in the thermal mixer to 56°C.

8. **Gently pellet spheroplasts by centrifugation for 3 minutes at 300 x *g*, then remove the supernatant**.

9. **Using a 1000 µl wide-bore pipette tip, gently resuspend the spheroplast pellet in 150 µl of cold 0.5 M NaCl**.

PART 2 YEAST CELL LYSIS

1. **Prepare a master mix of 450 µl HMW gDNA Tissue Lysis Buffer and 15 µl Proteinase K per sample.**

2. **Add 450 µl of lysis buffer master mix (Tissue Lysis Buffer + Proteinase K) to the sample and pipette thoroughly to mix using a wide-bore 1000 µl pipette tip.** If processing multiple samples, place each sample in the thermal mixer (Step 3) immediately after mixing with lysis buffer master mix.

3. **Incubate at 56°C for 30 minutes in thermal mixer with agitation at 2000 rpm**. The lysate should become mostly clear as the incubation proceeds.

4. **Add 10 µl RNase A and mix by inverting 5-10 times. Incubate for 10 min at 56°C in a thermal mixer with agitation at 2000 rpm.** For yeast samples, the amount of RNase A added, and incubation time may require additional optimization to reduce RNA contamination in purified HMW DNA.

5. **Centrifuge for 3 minutes at 16,000 x *g* to pellet cellular debris. Using a 1000 µl wide-bore pipette tip, transfer the supernatant to a new 1.5 ml microfuge tube**.

6. **Add 0.5 volume (~300 µl) of Protein Separation Solution and mix by inverting for 1 minute**.

7. **Centrifuge for 10 minutes at 16,000 x *g*. If working with multiple samples, during centrifugation, prepare the plastics for Part 3, as indicated in the following step.** The sample will separate into a large, clear upper phase (DNA) and a lower, clear phase (protein, usually on the bottom of the tube). A small amount of residual cellular debris may also be visible at the bottom of the tube.

8. **If working with multiple samples, prepare and label the plastics for the upcoming steps**. Each sample will require:
    - 1 Monarch Spin Collection Tube (no need to label)
        - 1 Monarch Bead Retainer inserted into the collection tube; this will be used to remove the wash buffer from the gDNA bound to the beads.
    - 2 Monarch 2 ml Tubes; one for phase separation and one for elution.
        - 1 1.5 ml microfuge tube (DNA low bind recommended, not provided); this will be used to collect the eluate.

9. **Using a 1000 µl wide-bore pipette tip, transfer the upper phase containing the DNA (large, clear phase) to a labeled Monarch 2 ml Tube.** Use a 200 µl wide-bore pipette tip to transfer any remaining volume of upper phase. Avoid transferring material from the protein layer at the bottom of the tube, or visible protein material that may be floating in the upper phase. Typically, the transferred volume will be ~ 800 µl.

PART 3 HMW gDNA BINDING AND ELUTION

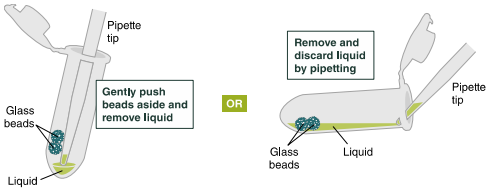
1. **Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube**.

2. **Add 0.7 volumes isopropanol, close the cap, and mix on a vertical rotating mixer at 10 rpm for 5 minutes to attach DNA to the beads**.

    If a vertical rotating mixer is not available, invert slowly and gently by hand 30 times. A manual inversion is complete when the tube returns to the upright position. Slow inversion is critical for the DNA to bind to the beads; each full inversion should take ~5–6 seconds. If necessary, flick the tube to release any beads that stick to the bottom of the tube.

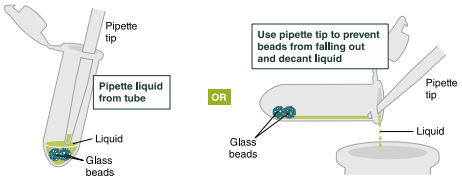
    After a 2–3 inversions, the solution becomes more viscous and the DNA will wrap loosely around the beads. During the following inversions, precipitation of gDNA may be visible, especially with larger input samples. The DNA complex will often contain small air bubbles. With increasing number of inversions, the DNA will completely wrap around the beads, often causing the beads to stick together. DNA binding to the beads should be complete after 25–30 inversions, and the solution should no longer be viscous. Additional inversions may be necessary for larger input samples.

3. **Remove and discard liquid by pipetting. Avoid removing any of the gDNA wrapped around the glass beads. For optimal DNA solubility, avoid letting the bound DNA dry out on the beads during this and the following steps; add the next buffer quickly.** There are two suggested options for carrying out this step:
    - Keeping tube upright, insert pipette tip and gently push beads aside to remove liquid.
    - Angle tube so that beads remain at the bottom, and liquid reaches toward tube opening. Pipette from the liquid surface and continue to angle as liquid is removed (tube will be almost horizontal at the end).

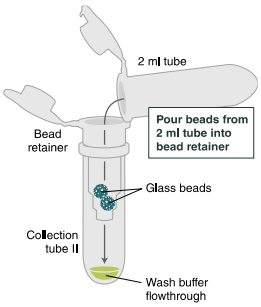


4. **Add 500 µl gDNA Wash Buffer, close the cap, and mix by inverting the tube 2–3 times. Remove the wash buffer as described in Step 3.** The loose gDNA complex will condense around the beads more tightly.

5. **Repeat the wash in Step 4 and remove the wash buffer by pipetting**. Alternatively, the buffer can be removed by decanting: position a pipette tip at the top of the angled tube to prevent the beads from falling out. It is not necessary to remove all the gDNA Wash Buffer at this point.

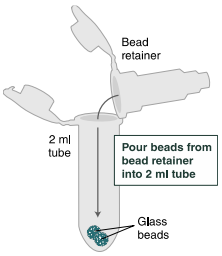


6. **Place a labeled Bead Retainer into a Monarch Spin Collection Tube. Pour the beads into the Bead Retainer and close the cap. Discard the used Monarch 2 ml Tube**. When working with multiple samples, be sure to close the cap of the Bead Retainer after each transfer of beads.

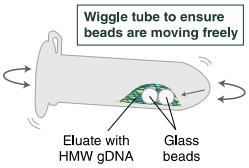


7. **Pulse spin (≤ 1 second) the sample in a benchtop minicentrifuge to remove any residual wash buffer from the beads**.

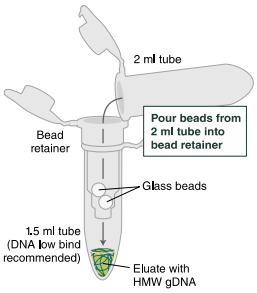
8. **Separate the Bead Retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube, and insert the used Bead Retainer into the labeled 1.5 ml microfuge tube for later use during elution. Discard the used collection tube.**



9. **Immediately add 100 µl Elution Buffer II onto the glass beads and incubate for a minimum of 5 minutes at 56°C in a thermal mixer with agitation at the lowest speed (300 rpm). Halfway through the incubation, ensure the beads are not stuck to the bottom of the tube by tilting the tube almost horizontally and gently shaking**. This ensures that the beads can move freely, allowing for optimal release of the DNA from the beads. It also ensures that the lower bead does not stick to the bottom of the tube during the following transfer step. Elution volume can be reduced to as low as 50 µl without affecting recovery. However, if using < 100 µl, the gentle shaking of the sample should be done several times during the incubation to ensure complete wetting of the beads.



10. **Ensure the Bead Retainer is inserted into the 1.5 ml microfuge tube (DNA low bind, not included). Pour the eluate and the glass beads into the Bead Retainer and close the cap**. When working with more than 1 sample, it is important to close the cap after each transfer of beads. Typically, all the eluate flows into the bead retainer upon pouring. If any volume remains in the 2 ml tube, spin briefly and transfer.



11. **Centrifuge for 30 seconds at 12,000 x g to separate the eluate from the glass beads. Discard the beads and retainer**.

12. **Pipette eluate up and down 5–10 times with a wide-bore pipette tip and ensure any visible DNA aggregates are dispersed. Before analysis or downstream use, HMW DNA must be homogeneously dissolved**. After pipetting, incubate at 37°C for 30-60 minutes, overnight at room temperature, or for > 24 hours at 4°C. Pipette up and down 5-10 times again before analyzing or using the HMW DNA. Samples processed using low agitation speeds during lysis will require additional time to fully dissolve. See additional guidance in "Homogenization of HMW DNA". Samples can be stored at 4°C for the short term (weeks) or at -20°C for long terms storage. The elution buffer (10 mM Tris, pH 9.0, 0.5 mM EDTA) is formulated for long term storage of gDNA.

Homogenization of HMW DNA Samples:

After elution, HMW DNA requires further manipulation before use and measurement. Eluted DNA will not be uniformly dispersed and often clumps in certain areas of the tube and requires time and effort to relax and disperse in solution. Spectrophotometric measurements that are carried out immediately following elution will, therefore, not give an accurate indication of the amount of DNA present. Also, if DNA is not completely dissolved, it may be detected in the OD measurement as turbidity, which will result in reduced A_{260}/A_{230} values that may be falsely interpreted as impurity.

General Approaches

- **Pipetting with Wide-bore Tips**
  When the DNA is eluted from the beads, the bulk phase retains the wrapped conformation it had when attached to the bead. Pipetting up and down with a 200 µl wide bore pipette tip breaks this conformation and facilitates dissolving and dispersion.

- **Heat**
  Heat will reduce the viscosity of the DNA solution and significantly increase the speed of homogenization of HMW DNA solutions. Below are some additional guidelines when using heat:

  - Temperatures > 60°C are generally not recommended as they will lead to DNA denaturation and degradation.
  - Incubation at 56°C, the temperature used for elution, is appropriate only for short incubation times. Do not exceed 30 minutes.
  - Samples can be incubated at 37°C for several hours safely; DNA integrity will not be affected.
  - Incubation at room temperature overnight facilitates even homogenization of the DNA.
  - Incubation at 4°C for days or weeks will enable enough relaxation of HMW DNA.

  If samples are incubated at 37°C for homogenization, mixing the samples at low agitation speed (300 rpm) will increase homogenization efficiency.

- **Time**
  HMW gDNA needs time to relax and homogenize. It is generally not recommended to work with freshly eluted DNA unless significant effort is made to ensure even DNA resuspension. Letting a sample relax overnight or for several days facilitates homogenization. If possible, it is recommended that UHMW DNA is extracted several days or a week prior to being needed for downstream application.

- **Dilution**
  HMW gDNA should ideally be kept at a concentration of 100–200 ng/µl for easy handling and reliable analysis. As such, using 200 µl elution buffer is recommended for samples obtained from > 10 mg soft organ tissue (liver, kidney, spleen, etc.) or ≥ 3 x 10^9 bacteria. Viscosity of solutions decreases significantly with dilution. After dilution, samples will require additional homogenization by the procedures described below.

For Samples Agitated at Maximum Speed

When agitation is carried out at 2000 rpm during lysis, purified DNA solutions should be only moderately viscous. In order to homogenize these samples prior to downstream analysis or use, the following steps are recommended:

1. After elution, pipette up and down 5–10 times using a 200 µl wide-bore pipet; ensure any clumps of DNA are dispersed.
2. Incubate DNA samples for 30–60 minutes at 37°C.
3. Pipette up and down 5–10 times again using a wide bore pipette tip.
4. (Optional) Briefly vortex; short vortexing will not affect overall DNA size.

DNA is now ready for use in downstream applications and can now be handled with standard pipette tips. If samples are not immediately required for downstream use, incubate the sample overnight at room temperature or at 4°C for further homogenization.

For Samples Agitated at Low Speeds (UHMW DNA)

Samples isolated following agitation with low speeds are extremely viscous and require additional effort to relax and homogenize before analysis and use. When homogenized completely, samples will appear consistent throughout the tube and OD measurements will become more consistent. The following steps are recommended:

1. After elution, pipette up and down 5–10 times using a 200 µl wide bore pipette; ensure any clumps of DNA are dispersed.
2. Incubate DNA samples for 30–60 minutes at 37°C.
3. Pipette up and down 5–10 times again using the same wide bore pipette tip.
4. Repeat 1–2 times each day for at least 2 days.

If quantitation of UHMW DNA remains challenging following the above steps, needle shearing is recommended prior to spectrophotometric measurement. Assuming enough sample is available, transfer 30–50 µl to a new tube using a wide bore pipette tip. Needle shear this aliquot with a 26-gauge (26G) blunt end needle connected to a 1 ml syringe until the viscosity is clearly reduced; up to 20 times may be necessary. Avoid pulling up any air into the needle. If the sample is too viscous to enter a 26G needle, use a larger gauge blunt end needle (e.g., 20G).

Measuring & Analyzing HMW DNA Samples

High molecular weight genomic DNA is often viscous and challenging to handle and transfer volumes with accuracy. Before measurement, samples should be properly homogenized following the guidelines above. Homogenization prior to quantitation is particularly important if samples have been frozen for long term storage, where gDNA is unevenly distributed upon thawing. When measuring thawed samples, allow them to reach room temperature and homogenize to enable consistent measurements.

Samples prepared at high agitation speed are suitable for concentration and purity assessment on microvolume spectrophotometers (MVS) (e.g., Nanodrop®) after they have been homogenized. These samples should be briefly vortexed to ensure even distribution of the gDNA in the solution before quantitation; a short vortex will not shear DNA.

Samples obtained with low agitation speeds (UHMW DNA) tend to be less homogeneous and may be more challenging to handle and apply on MVS systems. For these samples, mix with a wide-bore pipette tip to ensure an even distribution of DNA within the sample before quantitation. UHMW DNA needs to be measured several times. It is recommended to repeat the measurement ~5 times with different aliquots, then average the results for a to estimate the sample concentration. Chip-based systems like Unchained Labs® Lunatic (formerly known as Trinean Dropsense 16) are not suitable, as the viscosity of the samples prevents proper movement in the channels of the chip. In such cases, shearing a part of the sample using a 26-guage blunt end needle will help reduce viscosity (see “For Samples Agitated at Low Speeds (UHMW DNA)”). Alternatively, a fluorescence-based quantitation system (e.g., Qubit®) can be used.

Spectrophotometric analysis of gDNA eluates can be used for assessing the quantity of the isolated gDNA by measuring the absorbance at 260 nm. As the Monarch HMW DNA Extraction Kit protocol efficiently removes RNA using a nuclei prep approach, 260 nm absorbance values provide an accurate indication of the amount of DNA present. Typically, modern micro volume spectrophotometers (e.g., Nanodrop) automatically calculate the DNA concentration by multiplying the measured absorbance value with the conversion factor, which is 50 (A_{260} = 1 = 50 µg/ml for dsDNA). Concentration measurements at 260 nm can be performed on most microvolume systems down to 1 ng/µl with acceptable accuracy. Below that concentration, the use of fluorescence measurement via Qubit or similar detection systems is recommended. Please note that the $A_{260/230}$ and $A_{260/280}$ ratios are typically not reliable below 20 ng/µl. If DNA is not

completely dissolved, it may be detected in the OD measurement as turbidity, which will result in reduced A_{260}/A_{230} values that may be falsely interpreted as impurity.

Analysis by standard gel electrophoresis or other electrophoretic methods (Labchip®, Bioanalyzer®, TapeStation®) may not provide suitable resolution to accurately assess the distribution of fragment sizes in the eluted sample. Typically, more than 80% of the material is ≥ 50 kb in length. Resolution of high molecular weight gDNA is best performed by pulsed-field gel electrophoresis on agarose gels. Faster methods that have some utility include pulsed-field capillary methods (e.g., FemtoPulse).

Appendices

Considerations for Loading Pulsed-Field Gels (PFG)

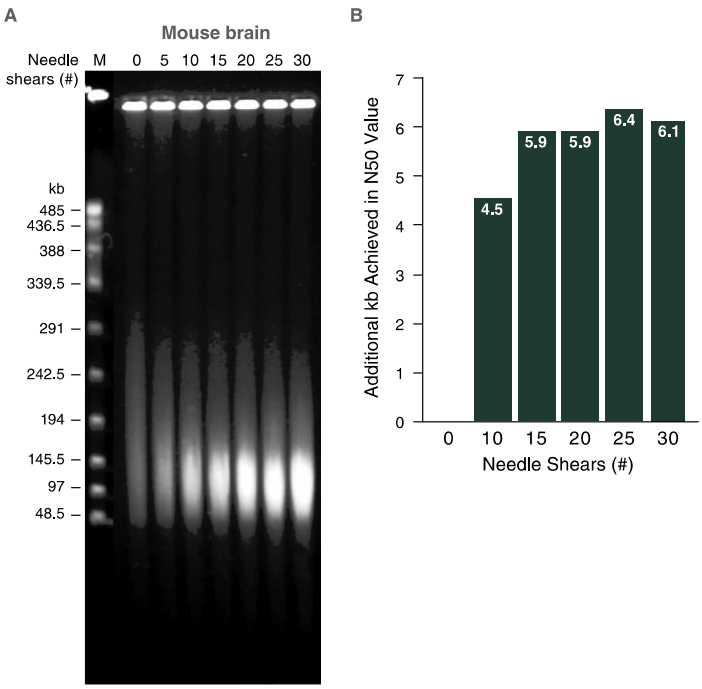
When samples are prepared for loading on pulsed-field gels, they are mixed with loading dye and water, and are thus diluted. As described above in "Homogenization of HMW DNA Samples", samples require time to disperse and relax following dilution. Failure to allow samples to fully relax before loading onto the gel can result in smearing and trailing of the DNA. As described, samples should be mixed by pipetting with a wide-bore pipette tip and incubated at 37°C for 30 minutes, or several hours to overnight at room temperature, after diluting with loading dye. Ensure that loading dye does not contain SDS, as SDS can cause the samples to float out of the well by introducing air bubbles into the sample. It is also recommended to use wide-bore pipette tips with hydrophobic coating, especially when working with UHMW DNA. These coated tips facilitate loading in the PFG wells by preventing the DNA from sticking to the pipette tip, which could otherwise lead to unintentionally pulling the DNA sample out of the well.

Considerations and Performance Data for Nanopore Sequencing

Successful Nanopore sequencing requires standard molecular biology processing including DNA repair, end-repair, adapter ligation, and cleanup. As such, the fragment length of material used is an important consideration during experiment design. According to internal testing, the use of the standard ligation-based Oxford Nanopore Technologies protocol with agitation at 2,000 rpm during lysis produced the highest N50 values, maximal percent of active pores on the flow cell, and maximal amount of data per run (often exceeding 10 Gb of data without flushing the flow cell). Using lower agitation speeds for longer DNA may seem logical since longer fragments should theoretically produce longer contigs. However, library prep of HMW DNA above a certain size has been shown to provide inferior results. It is recommended to target a DNA size range of 50–250 kb for optimal sequencing results.

- **Needle Shearing**
  DNA fragment length from pestle-homogenized tissue samples agitated at 2000 rpm often ranges up to 500 kb. As the optimal fragment length for standard ligation-based nanopore sequencing is 50-250 kb, the eluate can be passed through a 26-gauge blunt-end needle 10-20 times to reduce the fragment size.

Figure 5: Needle Shearing of HMW DNA Extracted from Tissue optimizes DNA fragment size range and improves N50 values in Nanopore Sequencing



200 µl of a pooled HMW genomic DNA sample from 3 x 20 mg mouse brain preps was needle sheared up to 30 times as indicated in the figure and resolved on a PippinPulse™ gel (Sage Science) with the program for 5–430 kb. M = Lambda PFG Ladder (NEB #N0341).

- **Loading Samples onto Flow Cells**
  Following library prep, consider loading additional DNA library onto the flow cell. Because the size of the DNA molecules extracted with this method are so long, more input material is needed to maintain the proper molar ratios. Loading 800–1500 ng of HMW gDNA on the flow cell provides optimal sequencing results in NEB's internal testing.

Table 2: Single Run Sequencing Results of Mouse Kidney Samples on the Oxford Nanopore Technologies Platform

Mean read length	27120.7
Mean read quality	13
Median read length	23150
Median read quality	13.5
Number of reads	164000
Read length N50	44631
Total bases	4447789727 (4.4 Gb)
Number, percentage and megabases of reads above quality cutoffs	
> Q5	164000 (100.0%) 4447.8 Mb
> Q7	163999 (100.0%) 4447.8 Mb
> Q10	148584 (90.6%) 4099.9 Mb
> Q12	124810 (76.1%) 3526.1 Mb
> Q15	12964 (7.9%) 305.3 Mb
Top 5 highest mean basecall quality scores and their read lengths	
1:00	18.7 (229)
2:00	18.7 (1041)
3:00	18.5 (792)
4:00	18.4 (200)
5:00	18.4 (550)
Top 5 longest reads and their mean basecall quality score	
1:00	200926 (12.3)
2:00	190512 (11.2)
3:00	182388 (14.5)
4:00	181646 (9.2)
5:00	177197 (13.0)

Single run ligation-based ONT sequencing with mouse kidney DNA extracted from fresh samples homogenized using a rotor-stator homogenizer. DNA used for the sequencing libraries was extracted by following the standard protocol for tissue with agitation at 2000 rpm. Libraries were generated using the LSK109 ligation sequencing kit and loaded on a FLO-MIN106D flow cell. Sequencing was performed on a GridION® Mk1 for up to 48 hours. Read lengths are indicated in bases unless otherwise noted.

Understanding Purity Ratios

Purity of the DNA samples can be assessed using A_{260}/A_{280} and A_{260}/A_{230} ratios. Samples that have A_{260}/A_{280} and A_{260}/A_{230} values > 1.8 are considered to be pure. However, when working with gDNA that is below 20 ng/µl, these ratios are no longer reliable. There is also great variation in the ratios depending on the device used to measure them.

A_{260}/A_{280} Ratio

A_{260}/A_{280} values can be used as a general guide for overall purity. For sufficiently concentrated samples (> 20 ng/µl), the following guidelines can be used:

- Mammalian gDNA samples that are very clean will show ratios at or near 1.85–1.87. The range 1.80–1.90 is generally considered clean.
- Values in the range 1.90 to > 2.0 may indicate potential RNA contamination. The higher the value, the greater the contamination level.
- Values < 1.80 may indicate potential protein contamination. The lower the value, the greater the contamination. In cases where the contamination is significant, a shoulder may be observed in the absorbance spectrum around 280 nm. It should be noted that the A_{260}/A_{280} ratio is only a rough indicator of protein contamination; low levels will not be detected as they would if using the A_{260}/A_{230} ratios.

A_{260}/A_{230} Ratios

A_{260}/A_{230} values can be used as a sensitive indicator for overall purity. There are many substances that may influence this ratio, therefore, analysis of A_{260}/A_{230} values should be performed with care. Moreover, A_{260}/A_{230} values show a higher coefficient of variation than A_{260} concentration values and A_{260}/A_{280} ratios, and their accuracy diminishes with decreasing analyte concentrations, particularly when measuring dilute samples with DNA concentrations below 20 ng/µl.

For DNA samples > 20 ng/µl, the following guidelines can be used:

- Samples that are very clean will show ratios in the range of 2.20–2.50. The range between 1.80–2.50 is generally considered clean.
- HMW DNA that is not fully dissolves causes sample turbidity, which is detected at 230 nm and will result in a lower A_{260}/A_{230} ratio
- The A_{260}/A_{230} ratio is a more sensitive indicator for protein contamination than the A_{260}/A_{280} ratio. Minor protein contamination will lead to lower A_{260}/A_{230} ratios (e.g., 1.60) but may have no significant effects on the A_{260}/A_{280} ratio.
- The presence of any form of aromatic molecules (like commonly used non-ionic detergents) or molecules with double bonds, (e.g., EDTA) will lower the A_{260}/A_{230} ratio.
- Traces of undigested hemoglobin from blood samples will give a specific absorbance peak at 410 nm. However, if measurable amounts of hemoglobin are available in samples stemming from blood material, the A_{260}/A_{230} ratio will also be affected.

Troubleshooting

Low DNA Yield

- Input amount too low
  - Use recommended input amounts for best results. If using an input amount below the recommended amount, DNA recovery will be significantly less efficient.
- Incomplete homogenization
  - Thorough tissue homogenization is necessary for optimal sample lysis. When processing tissue samples, work with the smallest possible tissue pieces to allow for efficient homogenization and rapid inactivation of nucleases by Proteinase K. Use the included pestle or a rotor-stator homogenizer to thoroughly homogenize tissue samples prior to incubation in the thermal mixer.
- Lysis volume too large
  - Use the appropriate lysis volume for the chosen input amount. The efficiency of binding to the glass beads is reduced when the DNA concentration is too low. Use of the appropriate lysis volume will establish optimal binding conditions and improve yields.
- DNA did not attach to beads
  - In rare cases, the DNA may not attach to the beads and remains in solution during the binding step. Twist the tube sideways to create contact between the precipitated DNA and the beads. If DNA still does not attach to the beads, spin the precipitate down and carefully remove the supernatant. Add 500 µl of Monarch gDNA Wash Buffer, carefully invert the tube 2-3 times, spin down briefly, and then remove as much wash buffer as possible, without disturbing the DNA pellet. Pulse-spin in a minicentrifuge and remove residual wash buffer. Next, invert the tube and air dry at room temperature for 5 minutes. Add elution buffer and incubate for 5-15 minutes at 56°C in a thermal mixer with agitation at the lowest speed (300 rpm). Pipette up and down with a wide-bore pipette tip to homogenize. Leave overnight at room temperature for further dissolving or incubate for 30 minutes at 37°C and mix by pipetting again.
- Incomplete binding to the beads
  - When working with high input samples, especially when low agitation speeds are used for UHMW DNA, additional time may be required for the DNA to bind completely and tightly to the beads. Increase the binding time on the vertical rotating mixer to 10 minutes. Insufficient binding time can lead to loss of DNA during the following short spin step.

- Inaccurate quantitation of UHMW DNA concentration
  - Measuring XL UHMW DNA accurately is challenging, as the large DNA molecules may be unevenly dispersed in solution. When measured on a spectrophotometer, the samples often show a significantly lower concentration than what is truly present. Repeated measurements may give a more accurate concentration estimate. Please refer to "Measuring & Analyzing HMW DNA Samples" for more information. Alternatively, shearing a small portion of the sample to reduce the viscosity and homogenize the solution as described in "Homogenization of HMW DNA Samples", on pages 27-29 to enable accurate quantitation.

- Incomplete transfer of upper phase during protein separation
  - In the protein separation step of the protocol, HMW gDNA is present as a gradient in the upper phase with the majority of the DNA being concentrated in the fraction closest to the protein phase. Transfer as much of the upper phase as possible for best yields.

- Shorter agitation time recommended for smaller input amounts
  - If using input amounts near the lower end of the input range, reduce agitation time from 45 minutes to 15 minutes during lysis. Finish the incubation time without agitation. This is particularly relevant for samples with low DNA content, like muscle and brain, and may increase yield by up to 50-100%.

Tissue:

- Sample was not stored properly
  - Fresh tissue samples should be processed immediately. If processing of fresh tissue samples is delayed for several hours, the quality of the isolated HMW DNA will be lower, particularly for metabolically active organ tissues.
  - Frozen samples should be kept frozen. In samples that have been frozen, ice crystals have destroyed cell structures and nucleases have free access to the genomic DNA. Snap freeze samples in liquid nitrogen to limit the damage to cell structures.

- Sample not sufficiently homogenized
  - When processing fresh or frozen tissue samples, work with the smallest possible tissue pieces to allow for efficient homogenization and rapid inactivation of nucleases by Proteinase K. Ensure tissue material is homogenized into the thinnest layer possible when using the pestle. If using a rotor stator homogenizer, homogenize sample until all visible tissue pieces are gone.

DNA Degradation

- Purified DNA sample kept at elevated temperatures too long
  - Although incubation at 56°C and 37°C helps to resuspend HMW DNA, particularly UHMW DNA, extended periods of heating HMW samples should be avoided, as this may result in DNA damage and eventually size reduction. Incubation time should not exceed 15–30 minutes for 56°C, 1–3 hours for 37°C and overnight incubation at room temperature. Samples are safe for storage at 4°C. Samples can be stored at 4°C for short term use; store at -20°C for longer term storage.

- Shearing introduced by inappropriate handling
  - UHMW DNA should always be pipetted using wide bore pipette tips and vortexing should be avoided. Extended heating at elevated temperatures will also negatively affect DNA fragment length. DNA samples purified using maximum agitation speeds have shorter fragment lengths; these can be pipetted with regular pipette tips and can be vortexed briefly.

Tissue:

- Sample was not placed in thermal mixer immediately following homogenization and addition of lysis master mix
  - Sample homogenization initiates endogenous nuclease activity. After pestle or rotor-stator homogenization, samples in lysis master mix should be placed in the thermal mixer immediately. When working with multiple samples, each sample should be taken quickly through all homogenization sub-steps and placed in the thermal mixer before processing the next sample.

- Sample was not stored properly

- Fresh tissue samples should be processed immediately. If processing of fresh tissue samples is delayed for several hours, the quality of the isolated HMW DNA will be lower, particularly for metabolically active organ tissues.
- Frozen samples should be kept frozen. In samples that have been frozen, ice crystals have destroyed cell structures and nucleases have free access to the genomic DNA. Snap freeze samples in liquid nitrogen to limit the damage to cell structures.
- When processing fresh or frozen tissue samples, work with the smallest possible tissue pieces to allow for efficient homogenization and rapid inactivation of nucleases by Proteinase K.
- Organ tissues like pancreas, intestine, kidney and liver contain significant amounts of nucleases. They should be treated with extreme care and stored properly to prevent DNA degradation. Keep frozen and on ice during sample preparation.

Protein Contamination

- Protein carried over after phase separation
  - When transferring the phase containing the DNA (large, clear upper phase) to a new tube, do not transfer any material from the protein phase. It may be necessary to leave ~10 µl of the DNA phase in the tube to prevent carrying over the protein phase. If a lower protein phase is not visible, leave ~30 µl behind to ensure protein is not carried over.
- Undissolved DNA falsely interpreted as protein contamination
  - If eluted DNA has not completely dissolved, this will result in the turbidity of the solution increasing, which will affect the A_{260}/A_{230} ratio. Upon complete dissolving of the DNA, the A_{260}/A_{230} will improve to values typically > 2.0 if the DNA is clean.

RNA Contamination

- Too much input material used, RNase A efficiency reduced
  - When sample input exceeds the recommended amounts, samples will be too viscous, resulting in reduced RNase A activity. Do not use more than the recommended input amount.

No Phase Separation

- Low input amount used
  - With low input amounts, a protein phase may not be visible after centrifugation and phase separation. Use input amounts indicated in the protocol for best results. If a lower protein phase is not visible, leave ~30 µl behind to ensure that you do not carry over protein.
- Protein is completely digested
  - In some cases, particularly when fresh tissue material is used, protein digestion may reach a high level of completion and no hydrophobic proteins will accumulate in a separate phase. Leave ~30 µl behind to ensure that you do not carry over protein.

Incomplete Phase Separation

- Too much starting material used
  - Follow the guidelines for tissue sample size. If the sample is not listed reduce input amount. Generally using more than 25 mg tissue is not recommended. For samples that are very DNA rich use no more than 15 mg.
- Low agitation speed used
  - Additional centrifugation time (10-20 minutes) may be required for complete phase separation when low agitation speeds are used.
- Fatty acid-rich samples need longer centrifugation time and prechilling
  - Pre-chilling lysates for 3 minutes on ice before adding Protein Separation Solution leads to more efficient phase separation. High input brain samples may require a longer centrifugation time for complete phase separation. Refer to “Special Considerations for Processing Specific Tissue Types: Brain”.

Low A_{260}/A_{230} (< 2.0) for Liver Samples

- Copurification of polysaccharides (i.e., glycogen) in liver preps results in variable or low A_{260}/A_{230} values. Addition of NaCl to a liver sample before DNA capture helps to remove polysaccharides, resulting in A_{260}/A_{230} values > 2.0. Refer to "Special Considerations for Processing Specific Tissue Types: Liver".

Ratio A_{260}/A_{230} > 2.5

- Slight variations in EDTA concentration in eluates:
  - If the EDTA available in the elution buffer complexes with magnesium or calcium cations, which may be associated with the isolated genomic DNA in small amounts, this will lead to small differences in the free EDTA concentration in the eluate. At NEB, we have observed EDTA has a strong influence on the 230 nm absorbance and a minute concentration reduction of free EDTA may lead to a higher-than-usual A_{260}/A_{230} ratio. In some cases, this ratio exceeds a value of 3.0 and is consistent with highly-pure samples. In these cases, the elevated value does not have any negative effect on downstream applications.

Eluted DNA difficult to dissolve

- DNA bound to glass beads was allowed to dry out
  - During washing and elution, it is important to prevent the bound DNA from drying out. Once a buffer is removed, add the next buffer quickly to keep the DNA wet. This will lead to optimal DNA solubility
- Too much input material used
  - If the suggested input amounts are exceeded, the optimal relationship between bead surface area and efficient wrapping of the DNA around the beads is not maintained. This can produce excessive compacting and tangling of the HMW DNA and inefficient resuspension of the DNA during the elution step. Reduce the input amount per the recommendations.
- Binding of DNA to the beads was carried out for too long
  - Do not exceed the recommended binding time as this will produce excessive compacting and tangling of the HMW DNA and lead to inefficient resuspension of the DNA during elution.

Low sequencing read lengths in ligation-based nanopore sequencing

- DNA is degraded: see "DNA Degradation" section above
- DNA Fragment Length is too large
  - For optimal results in ligation-based nanopore sequencing, the DNA fragments should be ~50–250 kb. If pestle homogenization was used, fragment lengths are often up to 500 kb. Needle shear 10–20X with a 26G blunt end needle as recommended in "Considerations and Performance Data for Nanopore Sequencing".

Ordering Information

PRODUCT	NEB #	SIZE
Monarch HMW DNA Extraction Kit for Tissue	T3060S/L	5/50 preps
Monarch Pestle Set	T3000S	100 sets
Monarch Bead Retainers	T3004L	100 retainers
Monarch DNA Capture Beads	T3005L	200 beads
Monarch Spin Collection Tubes	T2118L	100 collection tubes
Monarch HMW gDNA Tissue Lysis Buffer	T3061L	62 ml
Monarch Protein Separation Solution	T3062L	36 ml
Monarch gDNA Wash Buffer	T3015L	60 ml
Monarch gDNA Elution Buffer II	T3056L	20 ml
Monarch RNase A	T3018L	1 ml

Revision History

Revision #	Description	DATE
1.0	N/A	
2.0	Yeast protocol added. Additional guidance on processing large aliquots of frozen blood	2/21
2.1	Added guidance about preventing bound DNA from drying out on the beads	4/21
3.0	Updated kit components table with new component number. Updated formatting, header and footer. Updated legal.	3/24

How to Recycle Monarch Kit Components*

Component	Recycling Notes**
Kit Box (paper)	For the greatest environmental benefit, please reuse this box. It is fully recyclable in paper recycling. The small magnets do not prohibit recycling.
Columns and Collection Tubes (hard plastic)	Columns and collection tubes are made from polypropylene 5 and are recyclable. After use, please refer to your institutional policies for proper disposal, especially when working with biohazardous materials.
Plastic Bottles (hard plastic)	Bottles are made from high-density polyethylene 2, and caps are polypropylene 5. Please rinse before recycling.
Plastic Bags (plastic film)	Bags are made from low-density polyethylene 4 and can be recycled with other plastic bags and films.
Protocol Card (paper)	Recycle with mixed paper, or keep in your lab notebook for reference. The finish on this card does not prohibit recycling.

* Information as of November 2015. Please visit NEBMonarchPackaging.com for updates.
** Please defer to your institutional policies for proper disposal of this kit and its components.
Consult with your local and institutional authorities to learn how to maximize your landfill diversion and materials recovery.