INSTRUCTION MANUAL

Monarch® HMW DNA Extraction Kit for Cells & Blood

NEB #T3050S/L

5/50 preps

Version 3.0_03/24

Table of Contents

Kit Components

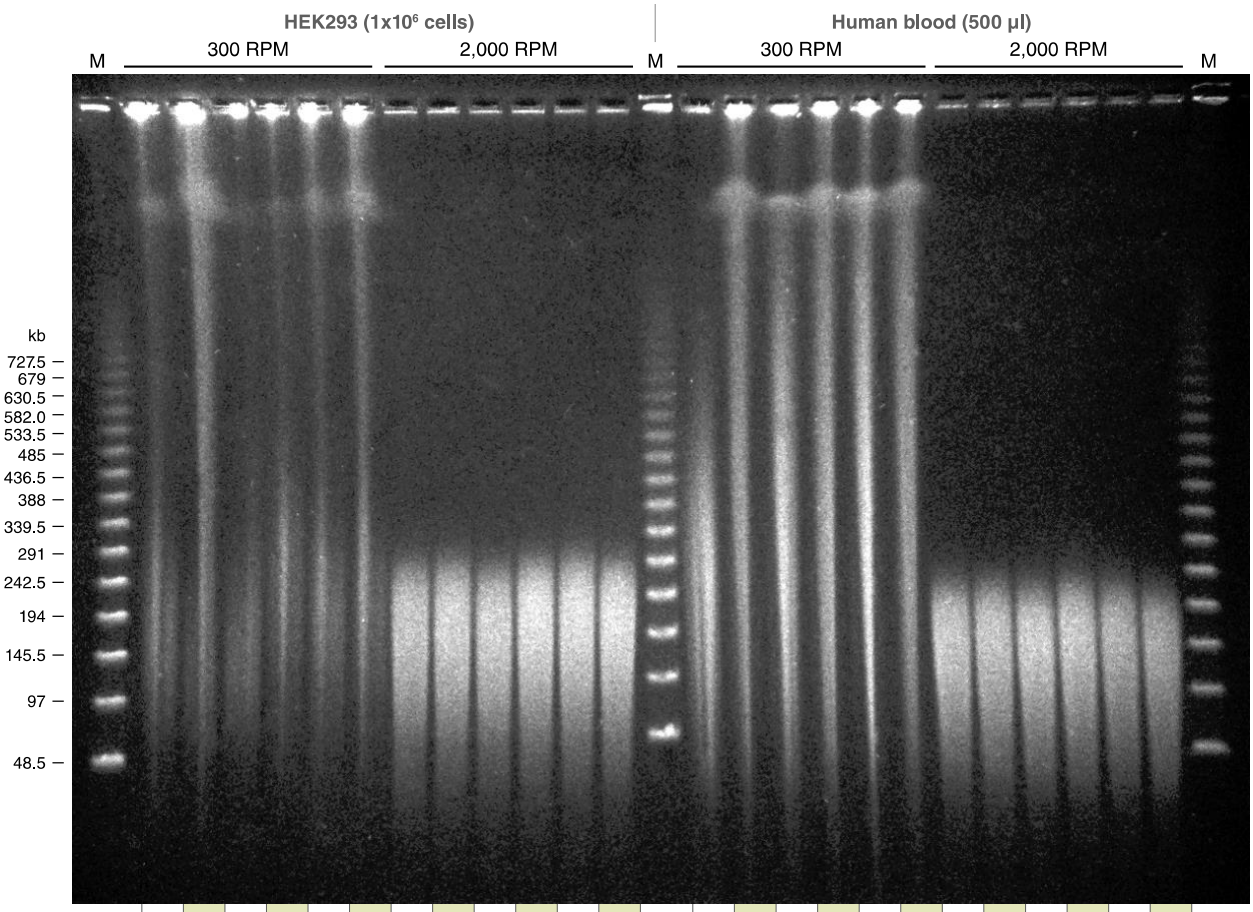
After opening the kit, RNase A and Proteinase K should be stored at -20°C and RBC Lysis Buffer should be stored at 4°C. The remainder of the kit should be stored at room temperature. Always keep buffer bottles tightly closed. For information regarding the composition of buffers, please consult the Safety Data Sheets available on our website (www.neb.com/T3050). Proper laboratory safety practices should be employed, including the use of lab coats, gloves and eye protection.

NEB #		T3050S 5 preps	T3050L 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
T2118	Monarch Spin Collection Tubes	5 tubes	50 tubes	25°C
T3051	Monarch RBC Lysis Buffer	15 ml	160 ml	4°C after opening
T3052	Monarch gDNA Nuclei Prep Buffer	1.5 ml	10 ml	25°C
T3053	Monarch gDNA Nuclei Lysis Buffer	1.5 ml	10 ml	25°C
T3055	Monarch Precipitation Enhancer	1 ml	5 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	35 µl	2 x 170 µl	-20°C after opening
P8200	Proteinase K, Molecular Biology Grade	120 µl	0.6 ml	-20°C after opening

Introduction

The Monarch HMW DNA Extraction Kit for Cells & Blood provides a rapid and reliable process for extracting high molecular weight (HMW) intact genomic DNA from cultured cells and whole blood. Utilizing an optimized process that combines gentle cell lysis with tunable fragment length generation followed by precipitation of the extracted DNA onto the surface of large glass beads, the prep proceeds rapidly and utilizes standard laboratory equipment. DNA size ranges from 50–250 kb for the standard protocol and can be adjusted to produce longer DNA into the megabase (Mb) range. Purified DNA is recovered in high yield with excellent purity, including nearly complete removal of RNA. For cells, the process time is only 30 min, while blood samples require erythrocyte lysis and are processed in 60 minutes. Purity ratios of 1.80–1.90 and 2.2–2.54 (A_{260}/A_{280} and A_{260}/A_{230}, respectively), are easily and reproducibly achievable, and purified HMW gDNA is suitable for a variety of downstream applications including long-read sequencing.

Figure 1: Reproducible Extraction of HMW DNA from Cells and Blood with the Monarch HMW DNA Extraction Kit.



Yield (µg)	11.9	13.6	12.8	14.3	14.2	13.6	11.3	11.1	10.9	12.1	11.5	11.7		15.5	14.4	13.4	15.2	13.2	13.8	15.0	13.5	12.7	13.8	14.2	13.4
260/280	1.87	1.87	1.86	1.86	1.86	1.87	1.87	1.88	1.88	1.88	1.87	1.88		1.87	1.84	1.85	1.87	1.85	1.87	1.86	1.85	1.86	1.86	1.87	1.87
260/230	2.38	2.49	2.47	2.48	2.38	2.49	2.51	2.42	2.47	2.45	2.51	2.46		2.56	2.47	2.52	2.49	2.45	2.57	2.41	2.41	2.40	2.51	2.32	2.49

DNA extracted with Monarch HMW DNA Extraction Kit for Cells & Blood. 1 x 10^6 fresh HEK293 cells and 500 µl fresh human blood were used as inputs and for preps performed according to the kit instructions using the agitation speed indicated above the gel lanes. 500 ng of DNA from the replicates 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 molecular weight standard.

Workflow

Specifications

Input	Cells: • Standard Input: > 5 x 10^5 to 1 x 10^7 • Low Input: 1 x 10^5 to ≤ 5 x 10^5 Blood: • Standard Input: 500 µl – 2 ml • Low Input: 100 µl – < 500 µl
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" pages 8-10. Typical protocols yield up to 125 µg of HMW DNA.
Genomic DNA Size	Using maximum agitation speed: 50 – 250 kb Using low agitation speed: up to and > 1 Mb
RNA Content	Less than 1.5%
Purity	OD $_{260/280}$ typically 1.8 – 1.9; $_{260/230}$ typically 2.2 – 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 Cell Samples

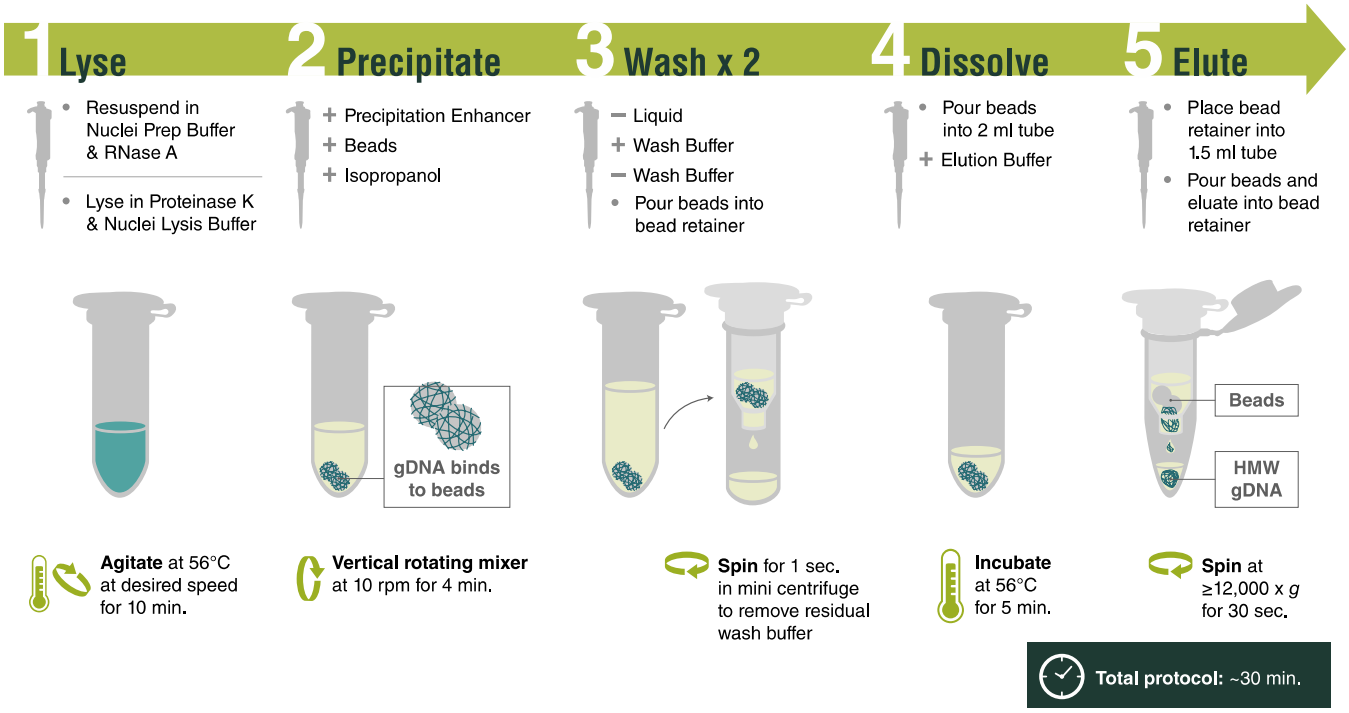
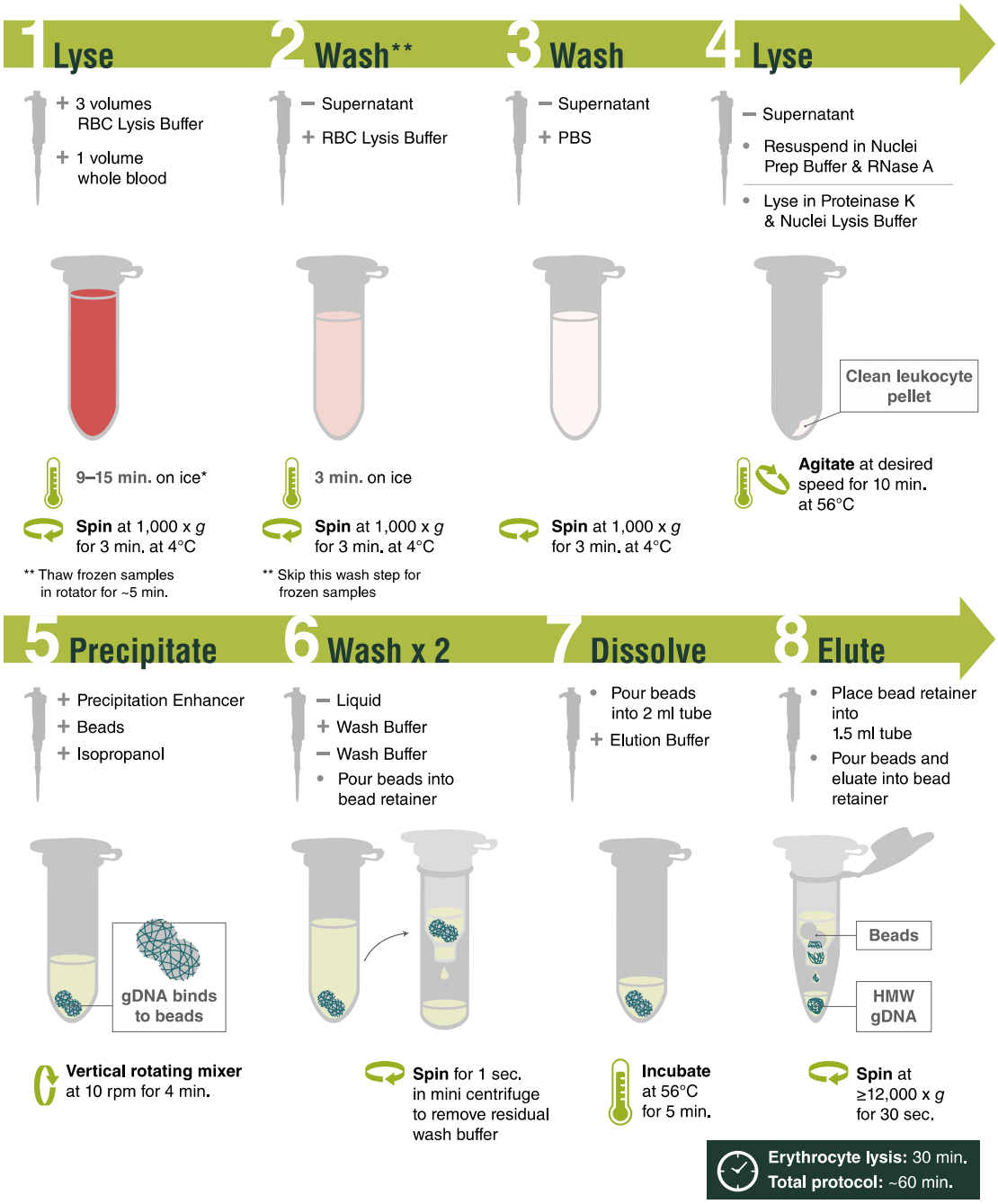


Figure 3: Workflow for Fresh Blood Samples



Principles of the Monarch HMW DNA Extraction Kit for Cells & Blood

This kit employs a novel approach to extraction of HMW DNA from cells and blood 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

Effective and efficient lysis of biological samples is an integral step in any nucleic extraction workflow. This kit employs a separate cytosolic lysis step followed by a nuclei lysis step to provide maximal purity, yield and DNA fragment size. For blood samples, erythrocyte lysis is carried out prior to leukocyte lysis in order to remove hemoglobin and maximize purity. The lysis buffers are optimized to provide protection against nucleases and allow for efficient and complete degradation of contaminating protein, eliminating the need for a separate protein removal step. In addition, the DNA fragment size can be tuned based on the agitation speed that is used during the lysis step.

Erythrocyte Lysis for Blood Samples

For blood samples, erythrocytes are initially lysed, and leukocytes are subsequently isolated with a simple pelleting step. Effectively, the content of the RBCs (including hemoglobin) and the content of the plasma (including many nucleases) are removed with the supernatant before the pelleted leukocytes are lysed. Purified leukocytes can then be processed similarly to cultured cells, and high quality, highly pure HMW DNA can be isolated. An initial erythrocyte lysis step also enables the scalability of the prep, allowing 100 µl to 2 ml of blood to be processed at one time, significantly increasing the yield of HMW DNA when compared to approaches that employ direct lysis of whole blood. Erythrocyte lysis works well for fresh and for frozen whole blood samples, and detailed guidance is provided for both options.

Nuclei Preparation and Lysis

Cultured cells or leukocytes from blood are gently lysed in two steps. Initially, cell membranes are lysed by the mild Nuclei Prep Buffer, leaving the nuclei intact and the HMW genomic DNA untouched and compacted inside. Lysis of the cell membranes enables cytosolic RNA to be digested by RNase A, which has been premixed with the Nuclei Prep Buffer. Subsequently, the nuclei are lysed by the Nuclei Lysis Buffer + Proteinase K, releasing the HMW gDNA into the lysate, and enabling digestion of cellular proteins.

For extraction methods that do not employ a separate nuclei lysis step, cell samples can become extremely viscous after lysis, which poses significant challenges. Highly viscous samples are not only difficult to handle, but the viscosity hinders the ability of enzymes like RNase A to work, drastically decreasing their efficiency, thus resulting in high levels of RNA contamination. The two-step lysis approach in this protocol results in nearly complete removal of RNA and establishes conditions for tunable fragment length generation.

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 intact size of the recovered gDNA (300– ≥ 1000 kb). Faster agitation rates during processing introduce small amounts of shear force, reducing DNA fragment size to 50–250 kb (ideal for ligation-based sequencing); see Choosing Agitation Speed During Lysis).

DNA Capture

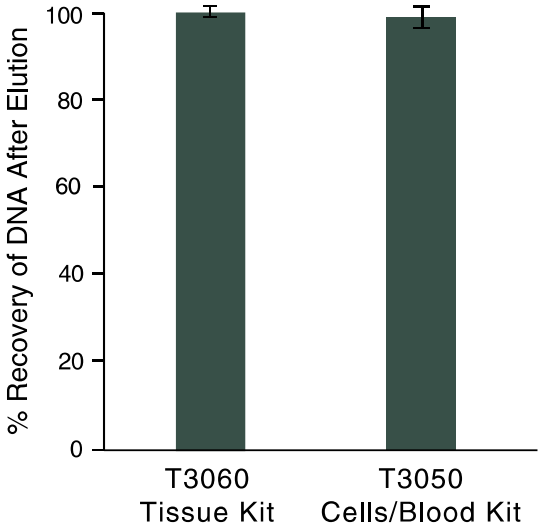
DNA extraction methods that employ magnetic beads or spin columns lead to shearing and typically only allow for the purification of DNA up to 150–200 kb. In the Monarch HMW DNA Extraction Kit, alcohol-based precipitation facilitates attachment of the genomic DNA to specialized glass beads, which provide 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 100 µg of DNA, is limited by the working volumes of the reagents and by the potential increases of viscosity that can result with 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 that buffer are rapidly removed. The efficient lysis procedure, coupled with this wash step, effectively removes lipids, proteins and salts, resulting in a streamlined purification without the need for a separate protein removal step.

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

Mammalian Cultured Cells:

HMW gDNA can be extracted from fresh and frozen cultured cells (adherent or suspension). Fresh cells contain high levels of RNA, but the two-step lysis approach described above ensures that RNA is efficiently degraded before the gDNA is released into the lysate and before Proteinase K, which degrades the RNase A enzyme, is introduced. Typically, yields from fresh samples range between 9 and 13 µg for 1 x 10^6 cells, which is significantly higher (typically ~50%) than yields typically obtained with silica spin columns.

For storage of cell culture samples, it is recommended to prepare aliquots of the desired cell count, pellet by centrifugation, shock freeze with liquid nitrogen and store at -80°C. Snap freezing, as opposed to slower freezing, ensures that cell structures remain intact and are only minimally affected by the freezing process. This will lead to maximal DNA integrity

and better results (read length) in ligation-based nanopore sequencing. Yield and purity of frozen cell samples will be comparable to those of fresh cell samples.

Cell samples that are stored in stabilization reagents (e.g., Monarch DNA/RNA Protection Reagent, RNAlater®) can also be processed, though it is recommended to work with fresh or frozen samples without stabilization reagents for optimal yield, purity and longer, more uniform DNA fragment size.

For samples stored in RNAlater, the cells are still intact and not yet lysed. Pellet the cells, carefully remove the RNAlater, and then follow standard protocol. For samples stored in 2X Monarch DNA/RNA Protection Reagent, cells are already lysed. Dilute the reagent to 1X with nuclease-free water and use this in place of the Nuclei Prep and Lysis Buffer combination for sample lysis. Following the 10-minute incubation with Proteinase K, add RNase A and incubate the sample at 56°C for 5 minutes. Subsequently, follow the standard protocol.

Blood:

Blood collected in the presence of common anticoagulants (EDTA, citrate and heparin) can be processed, and both fresh and frozen blood samples can be used. Blood preps are initiated with an erythrocyte lysis step, which helps obtain high yields, maximal purity, and high quality HMW DNA. However, during this process, extended exposure of leukocytes to the RBC Lysis Buffer results in a significant reduction of viability. Therefore, it is important to carry out the RBC lysis steps as quickly as possible and avoid any wait times between steps. Once the leukocytes are washed with PBS, their condition is stable.

- **Storage and Stabilization of Blood Samples**
  Regardless of the anticoagulant used, blood samples are processed effectively and without any notable differences in the results. When heparin is used, leukocyte pellets may be more difficult to resuspend, but yield and purity are unaffected. PAXgene® DNA Blood stabilization tubes provide excellent results for both fresh and frozen blood samples; leukocytes are efficiently stabilized in these tubes, and slightly higher overall yields have been observed when compared with other anticoagulants.

- **Fresh Blood Samples**
  It is preferable to work with fresh blood whenever possible. Blood sample quality decreases with each day of storage, and leukocyte stability, DNA yield, and length of the isolated DNA fragments will decline as a result. Additionally, as leukocyte stability declines, an increase in “stickiness” of the cells may be observed, requiring more effort to resuspend the pellet completely. Blood samples older than a week should not be used. Before processing, fresh blood samples should always be inverted several times or vortexed briefly to ensure an even suspension of the cell population.

- **Frozen Blood Samples**
  If blood samples need to be frozen for later processing, samples should be aliquoted immediately after collection in appropriately sized volumes/containers and should be snap frozen in liquid nitrogen. Frozen aliquots should accommodate the addition of 3 volumes of RBC Lysis Buffer; working in aliquots of 500 µl is recommended for most sample types and allows samples to be processed in 2 ml tubes. Blood samples should not be thawed completely unless in the presence of RBC Lysis Buffer, as DNA integrity will be compromised. If working with frozen blood samples aliquoted into volumes/containers that cannot accommodate the addition of RBC Lysis Buffer (e.g., vacutainers), see the “Important Notes for Frozen Blood” section of the protocol for specific guidance.

  During the freezing process, ice crystals damage the leukocyte cell walls, and upon thawing, cellular DNA becomes vulnerable to nuclease activity. Snap freezing ensures maximal viability of the leukocytes and will lead to higher yields, maximal DNA integrity and better results (read lengths) in ligation-based nanopore sequencing. Frozen samples collected in blood tubes with common anticoagulants (EDTA, citrate, heparin) should, therefore, always be thawed in the presence of cold RBC Lysis Buffer, which prevents damage to the gDNA. One exception is when working with samples frozen in PAXgene DNA Blood Tubes, where leukocytes are efficiently protected from nucleases. These samples do not require thawing in the presence of RBC Lysis Buffer; thawing can be performed according to the manufacturer’s guidance (37°C for 15 minutes), and samples can subsequently be aliquoted and mixed with cold RBC Lysis Buffer as indicated in the protocol.

  When processing frozen samples, it is important to use cold RBC Lysis Buffer and to move swiftly through the thawing process to limit the exposure of the cells to warm temperatures. It is also essential to resuspend any cell clumps that remain at the end of the thawing process by vortexing; cell clumps that are not resuspended will result in partially insoluble DNA at the end of the prep. Resuspension of leukocyte pellets from frozen samples is more challenging than with fresh samples, as previously frozen leukocytes have a stronger tendency to clump.

  Leukocytes frozen from freshly collected blood samples are robust and typically survive the freeze and thawing process quantitatively. However, leukocytes of aging blood samples exhibit decreased stability, which will result in reduced yields and reduced DNA fragment length. In some cases, using frozen samples instead of fresh samples may result in up to a 35% reduction in yield; in other cases, the difference may be minimal.

- **Mammalian Blood**
  Human blood samples can vary significantly among donors in terms of their erythrocyte and leukocyte content, as well as their leukocyte stability. Typically, DNA yields obtained from human samples range from 2.5 µg/100 µl for samples with low leukocyte counts to 6.5 µg/100 µl for leukocyte-rich samples.

  Working with animal blood is usually comparable to working with human blood. However, some animals, like horse, rabbit, and guinea pig have extremely high hemoglobin content, while others, like cow, have lower hemoglobin content; this is often noticeable in the dilute appearance of cow blood samples. Leukocyte content can also differ significantly, resulting in variability in DNA yields among different species. For example, pig and rhesus monkey samples typically yield high amounts of DNA (~8 µg and 10 µg per 100 µl, respectively), while cow and mouse samples typically yield lower amounts (1–2 µg per 100 µl).

  Rabbit blood is extremely rich in erythrocytes, posing a challenge for complete removal of hemoglobin and other proteins. For optimal purity, a reduced input of 200 µl is recommended. Erythrocyte lysis is not effective for frozen rat samples. Consequently, for rat blood, only fresh samples should be used. Leukocytes from goat and sheep blood samples are very fragile, making them unsuitable for erythrocyte lysis from frozen blood; for these, work only with fresh samples.

- **Nucleated Blood (birds, reptiles, fish)**
  Erythrocytes from non-mammalian vertebrates (birds, reptiles, fish) contain nuclei, and therefore have significantly higher DNA content. As such, input amounts should be reduced by 2 orders of magnitude compared to mammalian blood. Both fresh and frozen nucleated blood can be processed.

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 of 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 of 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 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 amounts, follow the detailed guidance that is provided.

Cells:

The sample input range is 1 x 10^5–1 x 10^7 cells, but an input amount of 1 x 10^6 cells is recommended. The upper limit for cell input amounts is dictated by the viscosity of the lysed sample, which poses a challenge to the efficiency of the enzymes, precipitation onto the beads, and the dissolving of the purified DNA. If using more than 2 x 10^6 cells, purified DNA samples will be viscous and may be more difficult to dissolve and handle. If using an input below 1 x 10^5 cells, DNA recovery will be significantly less efficient. It is important to note that if employing a low agitation speed during lysis, inputs should not exceed 5 x 10^6 cells. When working with samples less than 5 x 10^5, follow protocol guidance for Low Input to ensure the buffer volumes used reflect the lower cell count.

Blood:

- **Mammalian Blood**
  The sample input range for mammalian blood samples is 100 µl up to 2 ml. Working with a starting sample of 500 µl is recommended for most sample types; 200 µl is recommended for rabbit samples, which have a high cell content. If working with starting volumes > 500 µl, samples will need to be initially processed in larger tubes or split into 500 µl aliquots. The container needs to accommodate the addition of 3 volumes of RBC Lysis Buffer; working with 500 µl enables processing in a 2 ml tube. If splitting the sample into multiple aliquots, the pellets can be combined into a single 2 ml tube after RBC lysis to facilitate further processing. If working with sample volumes below the recommended input amount (500 µl for most samples), follow the protocol guidance for Low Input to ensure the buffer volumes reflect the lower cell count.

- **Nucleated Blood**
  The maximum input for successful DNA extraction from nucleated blood is 20 µl; exceeding this will overload the system. The recommended input amount is 5 µl.

- **Detailed Input Amount Guidance**
  Table 1 provides data on minimum, maximum, and recommended input amounts for various cell lines and blood samples using the Monarch HMW DNA Extraction Kit for Cells & Blood. Data on yield, purity, RNA content and DIN values is also provided. Samples that were successfully tested in standard ligation-based Oxford Nanopore Technologies sequencing runs are indicated. 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). Yields from blood samples vary by donor due to different leukocyte content; yield can vary up to 3-fold by donor. Similar yield and purity results were obtained with different anticoagulants (e.g., EDTA, citrate, heparin and PAXgene Blood DNA tubes were tested).

Using input amounts below the recommended minimums will reduce yields drastically. Exceeding maximum input amounts will result in DNA eluates that are highly viscous and difficult to dissolve and will reduce purity of the isolated DNA. Results are shown for samples that were lysed with agitation at 2,000 rpm.

Table 1: Guidance on sample input amount and expected results

Cells

	MINIMUM INPUT (CELLS)	MAXIMUM INPUT (CELLS)*	RECOMMENDED INPUT AMOUNT (CELLS)	YIELD (µg) FROM 1 x 10^6 CELLS	PURITY RATIOS		RNA CONTENT	VALIDATED FOR ONT SEQUENCING?
					A260/280	A260/230		
HEK293	1 x 10^5	1 x 10^7	1 x 10^6	11.5–13	1.86	2.4	≤ 1%	Yes
HeLa	1 x 10^5	1 x 10^7	1 x 10^6	12.9	1.86	2.4	≤ 1%	Yes
NIH3T3	1 x 10^5	1 x 10^7	1 x 10^6	9.4	1.86	2.4	≤ 1%	Yes
Jurkat	1 x 10^5	1 x 10^7	1 x 10^6	13.7	1.86	2.5	≤ 1%	Yes
K562 (suspension cells)	1 x 10^5	1 x 10^7	1 x 10^6	13.7	1.86	2.4	≤ 1%	Yes
HCT116	1 x 10^5	1 x 10^7	1 x 10^6	16.9	1.86	2.5	≤ 1%	Yes
A549	1 x 10^5	1 x 10^7	1 x 10^6	12.7	1.86	2.3	≤ 1%	Yes
U2Os	1 x 10^5	1 x 10^7	1 x 10^6	10.6	1.86	2.4	≤ 1%	Yes
HepG2	1 x 10^5	1 x 10^7	1 x 10^6	13.4	1.81	2.2	≤ 1%	Yes
NCI-460	1 x 10^5	1 x 10^7	1 x 10^6	9.5	1.86	2.4	≤ 1%	Yes
SK-N-SH	1 x 10^5	1 x 10^7	1 x 10^6	9.5	1.86	2.4	≤ 1%	Yes
Aa23	1 x 10^5	1 x 10^7	1 x 10^6	8.7	1.81	2.3	≤ 1%	Yes

Mammalian Blood

		MINIMUM INPUT (µl)	MAXIMUM INPUT (µl)	RECOMMENDED INPUT (µl)	YIELD (µg) for 500 µl**	PURITY RATIOS		RNA CONTENT	VALIDATED FOR ONT SEQUENCING?
						A260/280	A260/230		
Human***	Fresh	100	2,000	500	12–32	1.86	2.4	≤ 1%	Yes
	Frozen	100	2,000	500	9–30	1.86	2.4	≤ 1%	Yes
Mouse	Fresh	100	2,000	500	7–11	1.88	2.4	≤ 1%	Yes
	Frozen	100	2,000	500	16–17	1.88	2.4	≤ 1%	ND
Rat (fresh only)	Fresh	100	2,000	500	29–38	1.87	2.4	≤ 1%	Yes
Rabbit	Fresh	100	500	200	12–15	1.72	1.9	≤ 1%	Yes
	Fresh	100	500	200	200 µl: 4–5	1.89	2.4	≤ 1%	Yes
	Frozen	100	500	200	200 µl: 4–5	1.89	2.4	≤ 1%	Yes
Pig	Fresh	100	2,000	500	up to 42	1.86	2.4	≤ 1%	Yes
	Frozen	100	2,000	500	up to 40	1.86	2.4	≤ 1%	Yes
Horse	Fresh	100	2,000	500	16	1.86	2.3	≤ 1%	Yes
	Frozen	100	2,000	500	22.3	1.86	2.4	ND	ND
Cow	Fresh	200	2,000	500	7	1.86	2.4	≤ 1%	Yes
	Frozen	200	2,000	500	9.1	1.86	2.4	ND	ND
Rhesus monkey	Fresh	100	2,000	500	52	1.86	2.4	≤ 1%	Yes
	Frozen	100	2,000	500	52.6	1.86	2.5	ND	ND
Goat (fresh only)	Fresh	100	2,000	500	24	1.87	2.4	≤ 1%	Yes
Sheep (fresh only)	Fresh	100	2,000	500	15.3	1.87	2.4	ND	ND

Nucleated Blood

		MINIMUM INPUT (µl)	MAXIMUM INPUT (µl)	RECOMMENDED INPUT (µl)	YIELD (µg) per 5 µl	PURITY RATIOS		RNA CONTENT	VALIDATED FOR ONT SEQUENCING?
						A260/280	A260/230		
Chicken	Fresh	2	20	5	33	1.86	2.5	ND	Yes
	Frozen	2	20	5	30	1.86	2.5	ND	ND
Turkey	Fresh	2	20	5	37	1.87	2.4	ND	Yes
	Frozen	2	20	5	28	1.87	2.5	ND	ND

ND = Not determined

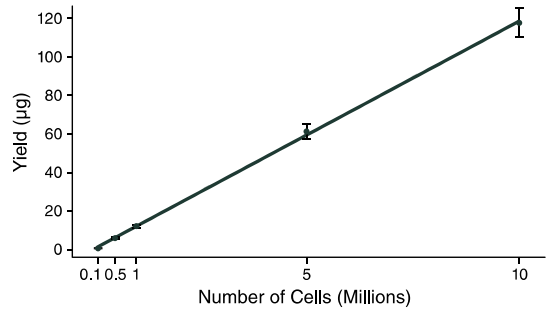
* For low agitation speeds, do not exceed 5 x 10^6 cells

** Unless otherwise stated

*** Compatible with K2-EDTA, Na-citrate, Na-heparin, PAXgene® Blood DNA

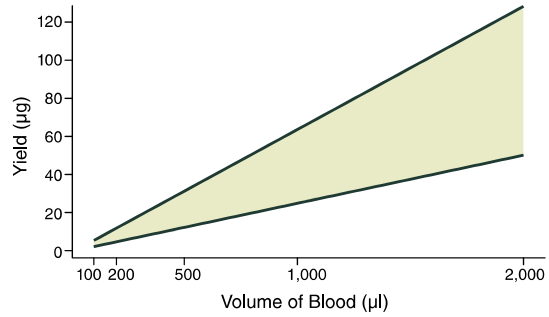
Figure 4: Linear correlation between yield and input for cells and blood.

A. HEK293 cells



Number of Cells	Typical Yield (µg)
$1x10^5$	0.5–1.0
$5x10^5$	5.5–6.5
$1x10^6$	11.5–13
$5x10^5$	57.5–65
$1x10^7$	110–125

B. Human blood



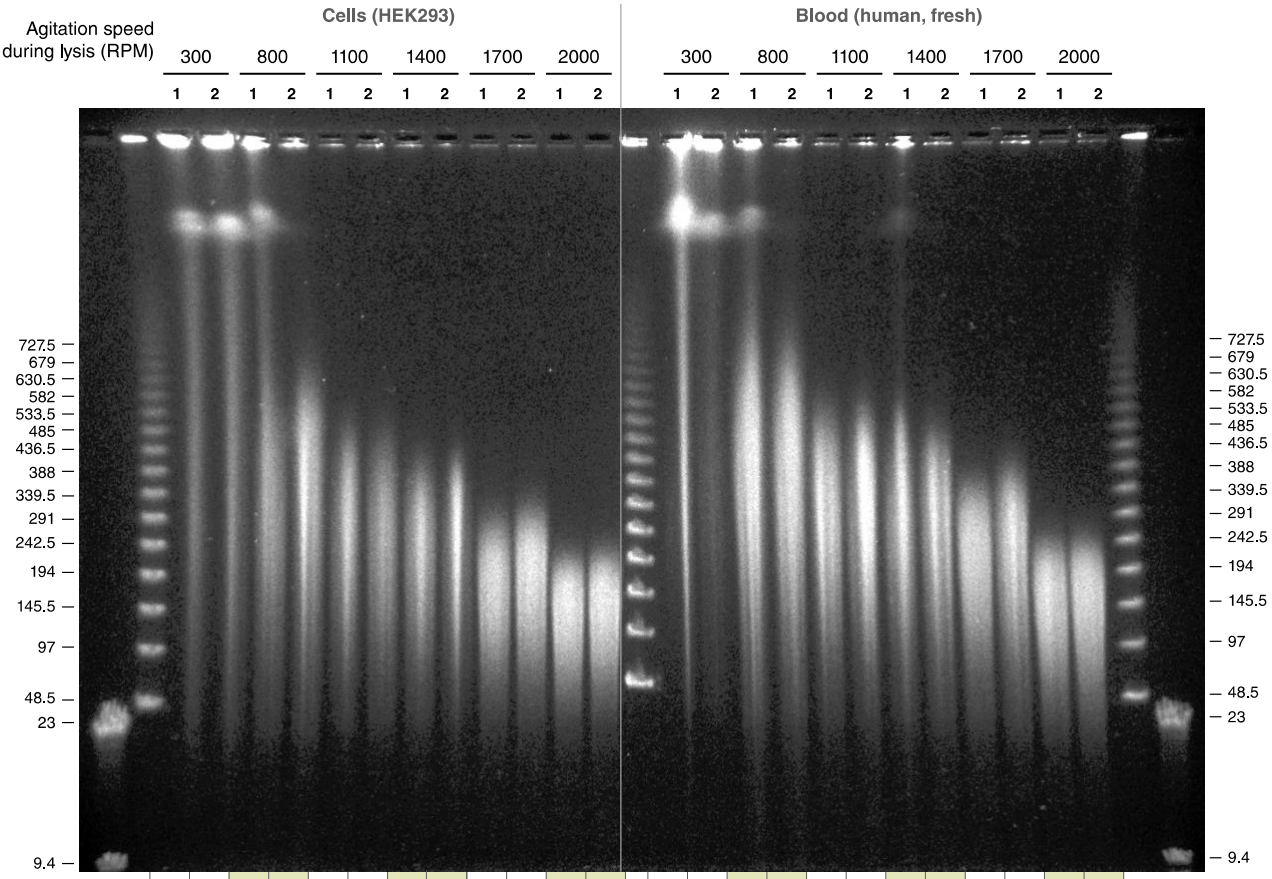
µl of Blood	Typical Yield (µg)	
	Low Leukocyte Content	High Leukocyte Content
100	2	5.1
200	4.4	11.2
500	12.5	32
1,000	25	64
2,000	50	128

Summarized yield data for HMW DNA preps are shown carried out at 2,000 rpm during lysis, using HEK293 cultured cells and fresh human blood samples from different donors as input material in the corresponding protocols. The starting materials were diluted to 5 different concentrations to cover the entire recommended input range. Cell samples ≤ 5 x 10^5 cells and blood samples <500 µl were purified using the recommended volumes for low input samples. Obtained yields show a high degree of linearity over the displayed input range.

Choosing Agitation Speed During Lysis

The Monarch HMW DNA Extraction Kit enables users to tune the size of extracted HMW 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 2,000 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 rpm or with no shaking, maximal fragment length, in the Mb range, will be obtained (UHMW DNA). These samples will be highly viscous and difficult to process.

Figure 5: Use of varying agitation speeds during lysis produces tunable fragment length of extracted HMW genomic DNA from cells and blood



	Cells (HEK293)											
Agitation speed during lysis (RPM)	300		800		1100		1400		1700		2000	
	1	2	1	2	1	2	1	2	1	2	1	2
Yield (µg)	11.3	11.6	11.1	12.1	11.7	12.4	13.2	12.0	12.7	12.9	12.0	13.5
260/280	1.86	1.85	1.87	1.87	1.87	1.87	1.88	1.87	1.87	1.86	1.87	1.87
260/230	2.31	2.28	2.36	2.44	2.39	2.47	2.49	2.50	2.52	2.53	2.48	2.44
DIN	9.7	9.8	9.8	9.8	9.9	9.8	9.8	9.7	9.8	9.8	9.8	9.7

	Blood (human, fresh)											
Agitation speed during lysis (RPM)	300		800		1100		1400		1700		2000	
	1	2	1	2	1	2	1	2	1	2	1	2
Yield (µg)	22.5	22.5	22.5	22.2	24.4	26.0	24.7	23.5	24.4	24.9	24.0	25.6
260/280	1.87	1.87	1.87	1.86	1.87	1.86	1.87	1.86	1.86	1.86	1.86	1.86
260/230	2.50	2.57	2.54	2.42	2.40	2.44	2.35	2.48	2.37	2.37	2.46	2.38
DIN	9.8	9.9	9.8	9.9	9.7	9.7	9.8	9.7	9.8	9.8	9.8	9.8

Preps were performed on duplicate aliquots of 1 x 10^6 HEK 293 cells and 500 µl fresh human blood. Samples were agitated at the indicated speed during the lysis step to control the fragmentation of the DNA. Equal amounts of DNA from the replicates (cells: 500 ng; blood: 650 ng) were resolved by PFGE (1% agarose gel, 6 V/cm, 13°C for 20 hours, switch times ramped from 0.5–94 seconds on a BioRad® CHEF-DR III System). Yield and purity ratios of the individual preps are shown in the accompanying tables. Lambda PFG Ladder and Lambda DNA-Hind III Digest (NEB #T3041 and #N3012) were used as molecular weight standards. Yield, purity ratios and DINs 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 manipulating 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 low DNA binding should be used. If an agitation speed of 2,000 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 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 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-HCl, pH 9.0, 0.5 mM EDTA) was 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 Cells

MATERIALS REQUIRED BUT NOT SUPPLIED

- Thermal mixer containing a 2 ml tube block (if not available, use a 1.5 ml block).
- Isopropanol, 275 µl per sample (Low Input: 100 µl/sample).
- Ethanol (≥ 95%)
- 1.5 ml DNase-free, low DNA binding microfuge tubes (e.g., Eppendorf® DNA LoBind®, #0030108051) are recommended 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.
- Recommended: vertical rotating mixer (e.g., Thermo Scientific® HulaMixer® Sample Mixer).
- Wide-bore pipette tips.

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.
- Cool the Nuclei Prep Buffer to 4°C.
- Preheat thermal mixer with 2 ml block to 56°C.

STARTING MATERIAL NOTES

- The sample input range is 1 x 10^5 –1 x 10^7 cells.
- An input of 1 x 10^6 cells is recommended.
- If using > 2 x 10^6 cells, purified DNA will be viscous and more difficult to dissolve and handle.
- If using 300 rpm for agitation, do not exceed 5 x 10^6 cells.
- Use the table below to determine the designation of your sample type, which will determine various volumes in the protocol.

PROTOCOL DESIGNATION	NUMBER OF CELLS
Standard Input	> 5 x 10^5 – 1 x 10^7; 1 x 10^6 is recommended. Do not exceed 5 x 10^6 cells if using 300 rpm agitation for "UHMW DNA"
Low Input	1 x 10^5 – < 5 x 10^5 Below 1 x 10^5 cells, DNA recovery is significantly less efficient.

HMW gDNA Purification Consists of Two Stages:

PART 1: Cell Lysis

PART 2: HMW gDNA Binding and Elution

PART 1 CELL LYSIS

1. **Pellet cells in a Monarch 2 ml Tube by centrifugation for 3 minutes at 1,000 x *g*.** Frozen pellets should be thawed.

2. **Prepare Nuclei Prep and Lysis Solutions as indicated below:**

   A. Nuclei Prep Solution: Combine cold Nuclei Prep Buffer and RNase A according to the table below and vortex to mix. Keep on ice.

	STANDARD INPUT		LOW INPUT	
# of SAMPLES	**VOLUME OF NUCLEI PREP BUFFER (µl)**	**VOLUME OF RNase A (µl)**	**VOLUME OF NUCLEI PREP BUFFER (µl)**	**VOLUME OF RNase A (µl)**
1	165	5.5	55	2
2	330	11	110	4
3	495	16.5	165	6
4	660	22	220	8
5	825	27.5	275	10

   B. Nuclei Lysis Solution: Combine Nuclei Lysis Buffer and Proteinase K according to the table below and vortex to mix. Keep at room temperature.

	STANDARD INPUT		LOW INPUT	
# of SAMPLES	**VOLUME OF NUCLEI LYSIS BUFFER (µl)**	**VOLUME OF PROTEINASE K (µl)**	**VOLUME OF NUCLEI LYSIS BUFFER (µl)**	**VOLUME OF PROTEINASE K (µl)**
1	165	11	55	4
2	330	22	110	8
3	495	33	165	12
4	660	44	220	16
5	825	55	275	20

3. **Flick to resuspend cell pellet. Add 150 µl (Low Input: 50 µl) of Nuclei Prep Solution and pipette up and down 10 times to mix, being careful not to introduce air bubbles. Incubate at room temperature for 2 minutes.** The sample will become less turbid, indicating cell lysis; nuclei remain intact.

4. **Add 150 µl (Low Input: 50 µl) of Nuclei Lysis Solution to sample and invert 10 times to mix. Avoid introducing air bubbles.** Do not vortex or pipette.

5. **Incubate at 56°C for 10 minutes in a thermal mixer with agitation at the desired speed to control the shearing and tune the size of gDNA.** The speed of the thermal mixer influences fragment length; higher speeds reduce overall size. For the standard ligation-based Oxford Nanopore Technologies (ONT®) sequencing protocol, agitation at 2,000 rpm is recommended. At 300 rpm or with no shaking, maximal fragment length, in the Mb range, will be obtained (UHMW DNA). These samples will be highly viscous and difficult to process. Optimization may be required depending on the quality of the starting sample. Refer to “Choosing Agitation Speed During Lysis”, page 12, for guidance. If desired, samples can be stored at 4°C overnight after the incubation.

6. **If working with multiple samples, prepare and label the plastics for Part 2: HMW gDNA Binding and Elution.**
   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.
   - 1 Monarch 2 ml Tube; this will be used for eluting the gDNA from the beads.
   - 1 1.5 ml microfuge tube (DNA low bind is recommended, not provided); this will be used to collect the eluate.

7. **Add 75 µl (Low Input: 25 µl) of Precipitation Enhancer after the 10-minute incubation and mix by inverting 8–10 times.**

PART 2 HMW gDNA BINDING AND ELUTION

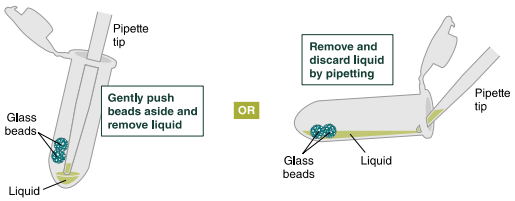
1. **Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube.**

2. **Add 275 µl (Low Input: 100 µl) isopropanol, close the cap, and mix on a vertical rotating mixer at 10 rpm for 4 minutes to attach DNA to the beads.** When working with ≥ 5 x 10^6 cells, double the inversion time to 8 minutes; this is especially important if low agitation speeds were used during lysis.

   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.

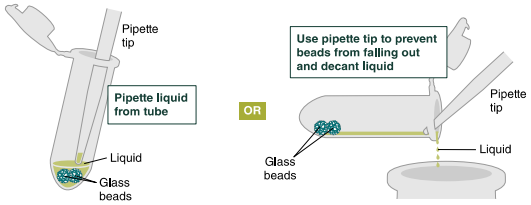
   After a few inversions, the solution becomes more viscous and the DNA will wrap loosely around the beads. During the following inversions, precipitation of gDNA may be visible, especially with sample inputs ≥ 5 x 10^6 cells. The DNA complex will often contain small air bubbles. With more inversions, the DNA will completely wrap around the beads, often causing the beads to stick together.

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).

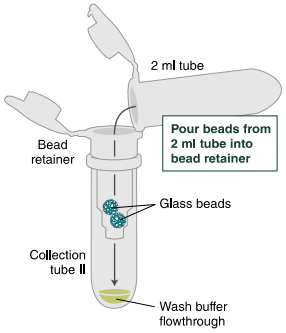


4. **Add 500 µl gDNA Wash Buffer, close the cap, and mix by inverting the tube 2–3 times. Remove the gDNA Wash Buffer as described in Step 3.** The loose gDNA complex will condense around the beads more tightly.

5. **Repeat the wash in Steps 3–4. 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.

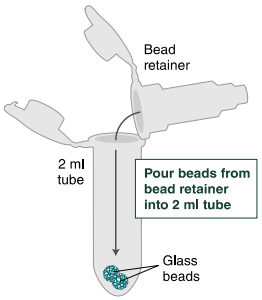


6. **Place a labeled bead retainer into a Monarch Spin Collection Tube. Pour the beads into the bead retainer and close the cap. Discard the used Monarch 2 ml Tube.** When working with multiple samples, be sure to close the cap of the bead retainer after each transfer of beads.

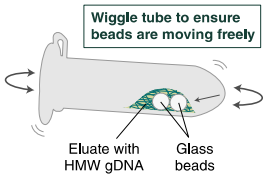


7. **Pulse spin (1 second or less) the sample in a benchtop minicentrifuge to remove any residual wash buffer from the beads.**

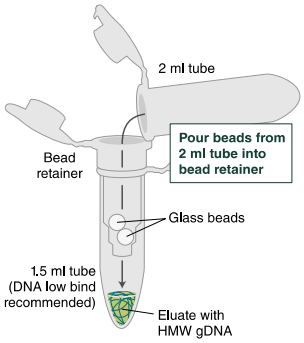
8. **Separate the bead retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube, and insert the used bead retainer into the labeled 1.5 ml microfuge tube (DNA low bind recommended, not provided) for later use during elution. Discard the used collection tube.**



9. **Immediately add 100 µl (use 200 µl if working with > 2 x 10^6 cells) Elution Buffer II onto the glass beads and incubate for 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. 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 (1 minute if working with ≥ 5 x 10^6 cells) 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”, page 24. 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 Blood

MATERIALS REQUIRED BUT NOT SUPPLIED

- Microcentrifuge capable of being set to 4°C. If working with blood aliquots > 500 µl, a centrifuge that accommodates 15 ml tubes will be required. Alternatively, split the sample into aliquots of 0.5 ml to use a microcentrifuge.
- Thermal mixer containing a 2 ml tube block (if not available, use a 1.5 ml block).
- Vertical rotating mixer (e.g., Thermo Scientific HulaMixer Sample Mixer) is recommended for all samples and is required for frozen blood.
- Ice or cooling block
- Ethanol (≥ 95%)
- Cold 1X PBS (1.5 ml per sample)
- Isopropanol, 275 µl per sample (Low Input: 100 µl per sample)
- 1.5 ml DNase-free, low DNA binding microfuge tubes (e.g., Eppendorf DNA LoBind, #0030108051) are recommended 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.
- Wide-bore pipette tips

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.
- Cool the Nuclei Prep Buffer, RBC Lysis Buffer and PBS to 4°C.
- Set centrifuge to 4°C.
- Preheat thermal mixer with 2 ml block to 56°C.
- ⚠ Once RBC Lysis Buffer has been added, proceed quickly through the protocol. Extended exposure of leukocytes to this buffer will lead to clumping and reduced DNA yield and quality.
- ⚠ It is essential to resuspend the cell pellet completely wherever indicated. Leukocyte clumping will lead to difficulty dissolving DNA.

STARTING MATERIAL NOTES

This protocol has been validated on fresh blood samples (human, mouse, rat, rabbit, horse, cow, pig and monkey) and frozen blood samples (human, mouse, rabbit, horse, cow, pig and monkey). Do not use frozen rat blood samples, as erythrocyte lysis is not effective; consider using a product employing direct lysis of whole blood (e.g., Monarch Genomic DNA Purification Kit (NEB #T3010). See “Important Considerations for Starting Materials”, pages 7-8 and “Choosing Input Amounts”, pages 8-10 before beginning, specifically if working with rabbit samples.

BLOOD SOURCE	PROTOCOL DESIGNATION	INPUT AMOUNT
Mammalian	Standard Input	500 µl–2 ml 500 µl is recommended*
	Low Input	100 µl to < 500 µl
Nucleated (e.g., bird, fish, reptile)	Standard Input	2–20 µl 5 µl is recommended

* If working with ≥ 500 µl blood, either split sample into 500 µl aliquots or work in a larger tube. If splitting samples into 500 µl aliquots, combine leukocyte pellets into 1.5 ml of RBC Lysis Buffer and transfer to a Monarch 2 ml Tube in Step 4 for further processing.

IMPORTANT NOTES FOR FRESH BLOOD

- Fresh samples provide the greatest yield; process blood samples soon after collection
- Leukocyte stability will decline after each day of storage. Cells will become stickier and difficult to resuspend.
- Mix blood sample well by vortexing before starting

IMPORTANT NOTES FOR FROZEN BLOOD

- If available, use liquid nitrogen to flash freeze blood samples.
- Freeze blood in aliquots of the desired volume immediately following collection to maintain integrity of leukocytes. Sample container needs to accommodate 3 volumes of RBC Lysis Buffer.
- Do not thaw the sample before processing, as DNA integrity will be compromised. Thaw in the presence of RBC Lysis Buffer, per the protocol.
- If samples have been frozen in containers which cannot accommodate 3 volumes of RBC Lysis Buffer (e.g., vacutainers), do not completely thaw the sample at room temperature or 37°C; samples should not be thawed unless in the presence of RBC Lysis Buffer. An effective approach is to start thawing the sample in a 37°C water bath just enough to detach it from its current container and move it to a larger one. Then add 3 volumes of cold RBC Lysis Buffer and continue the thawing process on the vertical rotator at 20 rpm until the blood has completely thawed (~3 minutes). Place on ice and take the appropriate amount for the DNA extraction (standard input is 500 µl blood, which equals 2 ml of the thawed blood + RBC Lysis Buffer mix).

HMW gDNA Purification Consists of Three Stages:

PART 1: Erythrocyte Lysis

PART 2: Leukocyte Lysis

PART 3: HMW gDNA Binding and Elution

PART 1 ERYTHROCYTE LYSIS

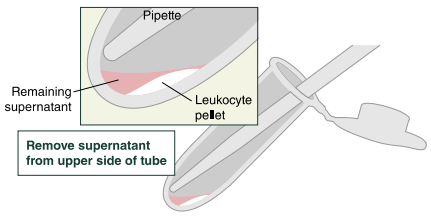
❑ **Fresh Mammalian Blood**

1. **Add up to 500 µl of blood to a Monarch 2 ml Tube and add 3 volumes of cold RBC Lysis Buffer (e.g., 1.5 ml RBC Lysis Buffer for 500 µl blood). Close the cap and invert 3–5 times to mix.** If working with rabbit blood, consider using an input volume of 200 µl; however, use 1.5 ml of RBC Lysis buffer instead of 3 volumes.

2. **Incubate on ice; when sample starts to turn translucent (6–12 minutes for fresh blood, 5–7 min for week-old blood), mix by inverting a few times, and then wait an additional 3–5 minutes for the RBC lysis to be complete.**

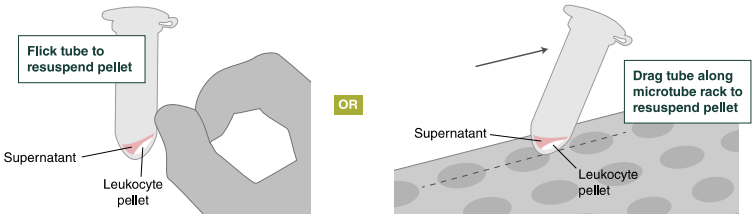
   ⚠ RBC lysis is stressful for leukocytes; it is important to process samples quickly; extended exposure of leukocytes to RBC Lysis Buffer can lead to clumping, lower yields and reduced solubility of the eluted DNA.

3. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then carefully remove most of the supernatant by pipetting, leaving approximately 15-20 µl behind to avoid disturbing the pellet. Close the cap.** Do not leave more than 20 µl behind, as this will lead to protein contamination and may interfere with DNA binding to the beads. Angle the tube, pellet down, and pipette from the upper side of the tube. Pipetting too close to the pellet will reduce DNA yield, as the pellet structure is loose.



4. **Flick the tube or drag along a microtube rack to resuspend the pellet. Briefly vortex sample and add 1.5 ml of cold RBC Lysis Buffer. Vortex to resuspend cells completely. Incubate on ice for 3 minutes.** Use 1.5 ml for all samples regardless of input amount. If working in a 15 ml tube, move sample to a Monarch 2 ml Tube after resuspension in RBC Lysis Buffer.

⚠ If working with multiple samples, resuspend, vortex, add buffer, vortex again and then move on to the next sample; this prevents clumping of the leukocytes.



5. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C. Remove supernatant by pipetting, leaving approximately 15-20 µl behind to avoid disturbing the pellet as described in Step 3, and close the cap.**

6. **Flick the tube or drag along a microtube rack to resuspend the pellet. Briefly vortex sample and add 1.5 ml of cold PBS. Vortex to mix.** If sample is not in a Monarch 2 ml Tube, transfer at this step.

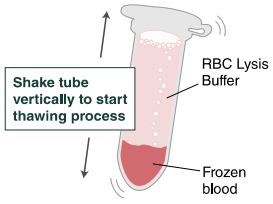
   ⚠ If working with multiple samples, resuspend, vortex, add PBS, vortex again and then move on to the next sample; this prevents clumping of the leukocytes.

7. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C and remove supernatant by pipetting, leaving approximately 15-20 µl behind as described in Step 3. Close the cap and proceed immediately to Part 2: Leukocyte Lysis.** Leukocyte pellets will be off-white and may contain a small residual amount of red blood cells. If pellet still contains a large fraction of red blood cells, repeat Steps 4-7. In the future, increase the incubation time in Steps 2 and 4 by a few minutes to ensure erythrocyte lysis is complete.

❑ Frozen Mammalian Blood

1. **Add 3 volumes of cold RBC Lysis Buffer, close cap and invert a few times, ensuring that the buffer can move freely within the tube.** If samples have been frozen in containers which cannot accommodate 3 volumes of RBC Lysis Buffer (e.g., vacutainers), see specific guidance in “Important Notes for Frozen Blood”, page 18.

2. **Incubate samples at room temperature for 30–90 seconds in a tube rack, shaking a few times to facilitate thawing, until RBC Lysis Buffer turns more intensely red.**



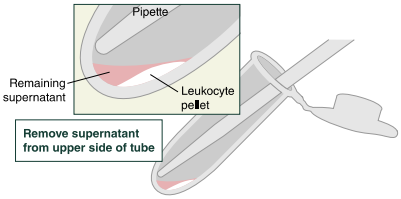
3. **Place samples in a vertical rotating mixer at ~10 rpm until pellet is thawed and dissolved. Do not exceed 5 minutes.** For 500 µl aliquots, thawing is usually complete after ~5 minutes. If after 5 minutes, the sample is not thawed, shake it a few times to facilitate complete thawing. Larger frozen aliquots may take longer to thaw. Remove from rotator *as soon as sample is thawed*, as extended exposure to warm temperatures will harm the leukocytes.

   Samples from some species, including rabbit, will form a very rigid frozen cell pellet that has a significantly higher cell count than other species. Support the thawing process by frequently flicking the tube vigorously to loosen the pellet. This ensures that thawing is complete within 5 minutes.

4. **After frozen blood has thawed completely, vortex to resuspend any remaining cell aggregates and keep on ice.**

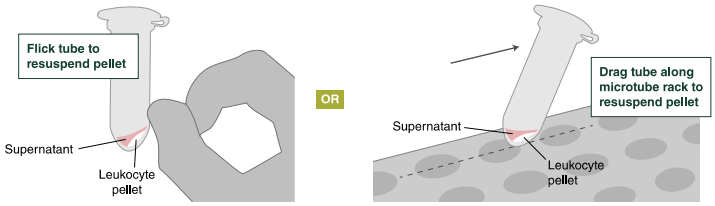
5. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then carefully remove most of the supernatant by pipetting, leaving approximately 15-20 µl behind to avoid disturbing the pellet. Close the cap.** Do not leave more than 20 µl behind, as this will lead to protein contamination and may interfere with DNA binding to the beads. Angle the tube, pellet down, and pipette from the upper side of the tube. Pipetting too close to the pellet will reduce DNA yield, as the pellet structure is loose.

   For samples with a low leukocyte content like cow blood or low input samples, there may not be a clearly visible pellet after centrifugation. However, leukocytes are present and spread more evenly along the tube wall. Process samples by removing the supernatant carefully while placing the pipette tip at the opposite side of the tube, to ensure the leukocyte layer is not disturbed.



6. **Vigorously flick the tube or drag along a microtube rack several times to completely resuspend the pellet. If sample has splashed along the tube walls, pulse spin in a benchtop minicentrifuge. Briefly vortex sample, add 1.5 ml of cold PBS, and vortex to mix.** If necessary, repeat vortexing to resuspend cells completely. If sample is not in a Monarch 2 ml Tube, transfer at this step.

   ⚠ If working with multiple samples: resuspend, vortex, add PBS, vortex again, and then move on to the next sample; this prevents clumping of the leukocytes.



7. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then remove supernatant by pipetting, leaving approximately 15-20 µl as described in Step 5. Close the cap, and immediately proceed to Part 2: Leukocyte Lysis.** If working with rabbit blood, repeat the PBS wash to reduce hemoglobin content.

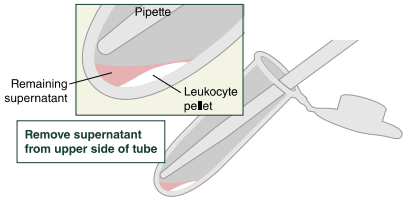
❑ Fresh Nucleated Blood

1. **Add 2–20 µl (5–10 µl is recommended) of blood to a Monarch 2 ml Tube and add 1.5 ml cold RBC Lysis Buffer. Close the cap and invert 3–5 times to mix.**

2. **Incubate on ice for 10 minutes; mix a few times by inverting during the incubation.** Sample will turn slightly translucent, but in contrast to non-nucleated blood, will not show a complete change from turbid to translucent.

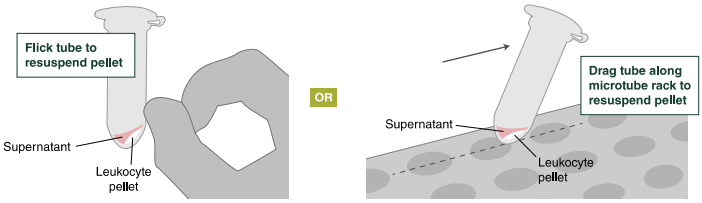
   ⚠ RBC lysis is stressful for leukocytes; it is important to process samples quickly; extended exposure of leukocytes to RBC Lysis Buffer can lead to clumping, lower yields and reduced solubility of the eluted DNA.

3. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then remove most of the supernatant by pipetting; leaving approximately 15-20 µl behind to avoid disturbing the pellet. Close the cap.** Angle the tube, pellet down, and pipette from the upper side of the tube. Pipetting too close to the pellet will reduce DNA yield, as the pellet structure is loose.



4. **Flick the tube or drag along a microtube rack to resuspend the pellet. Briefly vortex sample and add 1.5 ml of cold RBC Lysis Buffer. Vortex to resuspend cells completely and incubate on ice for 3 minutes.** Use 1.5 ml for all samples regardless of input amount. If working in a 15 ml tube, move sample to a Monarch 2 ml Tube after resuspension in RBC Lysis Buffer.

   ⚠ If working with multiple samples, resuspend, vortex, add buffer, vortex again and then move on to the next sample; this prevents clumping of the leukocytes.



5. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C. Remove supernatant by pipetting, leaving approximately 15-20 µl behind to avoid disturbing the pellet as described in Step 3, and close the cap.**

6. **Flick the tube or drag along a microtube rack to resuspend the pellet. Briefly vortex sample and add 1.5 ml of cold PBS. Vortex to mix.** If sample is not in a Monarch 2 ml Tube, transfer at this step.

   ⚠ If working with multiple samples, resuspend, vortex, add PBS, vortex again and then move on to the next sample; this prevents clumping of the leukocytes.

7. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C and remove supernatant by pipetting, leaving approximately 15-20 µl behind as described in Step 3. Close the cap, and immediately proceed to Part 2: Leukocyte Lysis.** Leukocyte pellets will be off-white and may contain a small residual amount of red blood cells. If pellet still contains a large fraction of red blood cells, repeat Steps 4–7. In the future, increase the incubation time in Steps 2 and 4 by a few minutes to ensure erythrocyte lysis is complete.

❑ Frozen Nucleated Blood

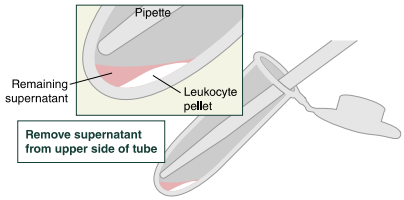
Before beginning, review the "Important Notes" relating to frozen blood samples. There are essential considerations that directly impact performance of the prep and the integrity of the DNA.

1. **Add 1.5 ml of cold RBC Lysis Buffer. Invert a few times until the blood starts to thaw.** RBC Lysis Buffer will begin to turn red.

2. **Incubate samples on ice 2–3 minutes, flicking periodically to facilitate thawing and resuspension.**

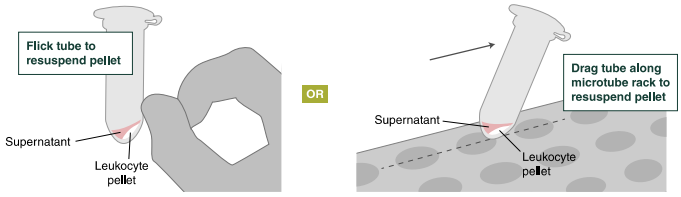
3. **After thawing is complete, vortex briefly to dissolve any cell aggregates.**

4. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then remove most of the supernatant by pipetting, leaving approximately 15-20 µl behind to avoid disturbing the pellet. Close the cap.** Angle the tube, pellet down, and pipette from the upper side of the tube. Pipetting too close to the pellet will reduce DNA yield, as the pellet structure is loose.



5. **Flick the tube or drag along a microtube rack to completely resuspend the pellet. If sample has splashed along the tube walls, pulse spin in a benchtop minicentrifuge. Briefly vortex sample, add 1.5 ml of cold PBS, and vortex to mix.** If necessary, repeat vortexing to resuspend cells completely. If sample is not in a Monarch 2 ml Tube, transfer at this step.

   ⚠ If working with multiple samples: resuspend, vortex, add PBS, vortex again, and then move on to the next sample; this prevents clumping of the leukocytes.



6. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C and remove supernatant by pipetting, leaving approximately 15-20 µl behind as described in Step 4. Close the cap, and immediately proceed to Part 2: Leukocyte Lysis.**

PART 2 LEUKOCYTE LYSIS

1. **Prepare Nuclei Prep and Lysis Solutions as indicated below:**

   A. Nuclei Prep Solution: Combine cold Nuclei Prep Buffer and RNase A according to the table below and vortex to mix. Keep on ice.

	STANDARD INPUT (500 µl – 2 ml blood)		LOW INPUT (<500 µl blood)	
# of SAMPLES	**VOLUME OF NUCLEI PREP BUFFER (µl)**	**VOLUME OF RNase A (µl)**	**VOLUME OF NUCLEI PREP BUFFER (µl)**	**VOLUME OF RNase A (µl)**
1	165	5.5	55	2
2	330	11	110	4
3	495	16.5	165	6
4	660	22	220	8
5	825	27.5	275	10

   B. Nuclei Lysis Solution: Combine Nuclei Lysis Buffer and Proteinase K according to the table below and vortex to mix. Keep at room temperature.

	STANDARD INPUT (500 µl – 2 ml blood)		LOW INPUT (<500 µl blood)	
# of SAMPLES	**VOLUME OF NUCLEI LYSIS BUFFER (µl)**	**VOLUME OF PROTEINASE K (µl)**	**VOLUME OF NUCLEI LYSIS BUFFER (µl)**	**VOLUME OF PROTEINASE K (µl)**
1	165	11	55	4
2	330	22	110	8
3	495	33	165	12
4	660	44	220	16
5	825	55	275	20

2. **Proceed with steps A-C for each sample before moving on to the next sample. Avoid introducing air bubbles when pipetting and mixing.**

    A. **Flick the tube or drag along a microtube rack to resuspend cell pellet completely.** If sample has splashed along the tube walls, pulse spin in a benchtop minicentrifuge.

    B. **Add 150 µl (Low Input: 50 µl) of Nuclei Prep Solution and pipette up and down 10 times (20 times for frozen samples) to mix.** The sample will become less turbid, indicating cell lysis; nuclei remain intact. Nuclei from frozen blood and from fresh blood that has been stored for several days may contain clumps. Pressing the pipette tip to the bottom of the tube during pipetting facilitates complete resuspension.

    C. **Add 150 µl (Low Input: 50 µl) of Nuclei Lysis Solution to sample and invert 10 times to mix.** Do not vortex or pipette. Repeat A-C with any remaining samples, leaving finished samples at room temperature.

3. **Incubate at 56°C for 10 minutes in a thermal mixer with agitation at the desired speed to control the shearing and tune the size of gDNA.** The speed of the thermal mixer influences fragment length; higher speeds reduce overall size. For the standard ligation-based Oxford Nanopore Technologies (ONT) sequencing protocol, agitation at 2,000 rpm is recommended. At 300 rpm or with no shaking, maximal fragment length, in the Mb range, will be obtained (UHMW DNA). These samples will be highly viscous and difficult to process. Optimization may be required depending on the quality of the starting sample. Refer to “Choosing Agitation Speed During Lysis, page 12, for guidance. If desired, samples can be stored at 4°C overnight after the incubation.

4. **If working with multiple samples, prepare and label the plastics for Part 3: HMW gDNA Binding and Elution.**
Each sample will require:
    - 1 Monarch Spin Collection Tube (no label needed).
    - 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.
    - 1 Monarch 2 ml Tube; this will be used for eluting the gDNA from the beads.
    - 1 1.5 ml microfuge tube (DNA low bind is recommended, not provided); this will be used to collect the eluate.

5. **Add 75 µl (Low Input: 25 µl) of Precipitation Enhancer after the 10-minute incubation and mix by inverting 8–10 times.**

PART 3 HMW gDNA BINDING AND ELUTION

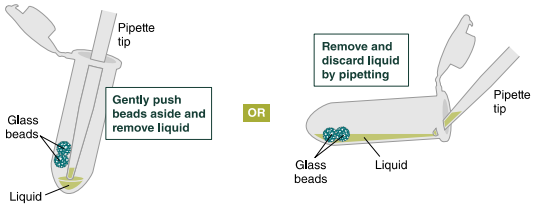
1. **Using clean forceps, add 2 DNA Capture Beads to each sample, which should be contained in a Monarch 2 ml Tube.**

2. **Add 275 µl (Low Input: 100 µl) isopropanol, close the cap, and mix on a vertical rotating mixer at 10 rpm for 4 minutes to attach DNA to the beads.** When working with ≥ 1 ml blood, double the inversion time to 8 minutes; this is especially important if low agitation speeds were used during lysis.

    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.

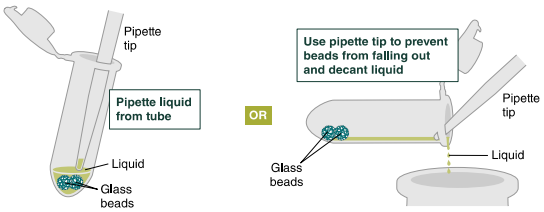
    After a few inversions, the solution becomes more viscous and the DNA will wrap loosely around the beads. During the following inversions, precipitation of gDNA may be visible, especially with sample inputs ≥ 500 µl. The DNA complex will often contain small air bubbles. With more inversions, the DNA will completely wrap around the beads, often causing the beads to stick together.

2. **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 gDNA Wash Buffer as described in Step 3.** The loose gDNA complex will condense around the beads more tightly.

5. **Repeat the wash in Steps 3–4. 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.

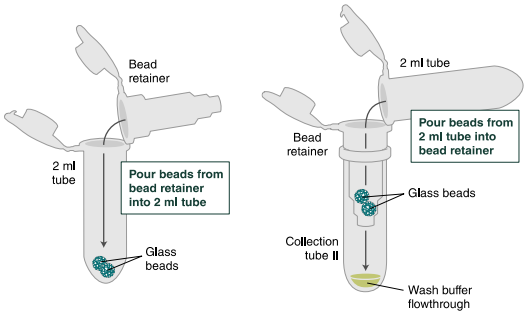


6. **Place a labeled bead retainer into a Monarch Spin Collection Tube. Pour the beads into the bead retainer and close the cap. Discard the used Monarch 2 ml Tube.**

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

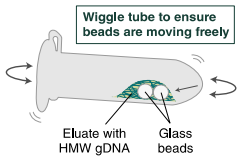
8. **Separate the bead retainer from the collection tube, pour the beads into a new, labeled Monarch 2 ml Tube, and insert the used bead retainer into the labeled 1.5 ml microfuge tube (DNA low bind recommended, not provided) for later use during elution. Discard the used collection tube.**

9. **Immediately add 100 µl (200 µl if working with ≥ 1 ml of blood or with ≥ 10 µl of nucleated blood) Elution Buffer II onto the glass beads and incubate for 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; do not let the liquid reach the cap.**



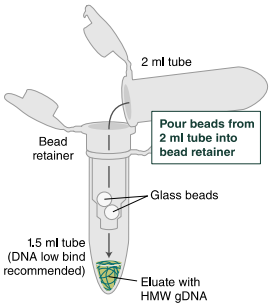
This ensures that the beads can move freely, allowing for optimal release of the DNA from the beads. It also ensures that the lower bead does not stick to the bottom of the tube during the following transfer step. Elution volume can be reduced to as low as 50 µl without affecting recovery. However, if 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. 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 (1 minute if working with ≥ 1 ml blood) 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”, page 24. Samples can be stored at 4°C for 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.



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; the DNA requires time and effort to disperse and return to its natural conformation 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 also facilitated homogenization and 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 concertation of 100–200 ng/ul for easy handling and reliable analysis. As such, using 200 µl elution buffer is recommended for samples obtained from > 2 x 10^6 cells, > 500 µl mammalian blood or > 5 µl nucleated blood. 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 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 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, as complete homogenization is challenging. It is recommended to repeat the measurement ~5 times with different aliquots, then average the results for a better estimation of the sample concentration. Chip-based systems like Unchained Labs® Lunatic® (formerly known as Trinean Dropsense16) are not suitable, as the viscosity of the samples prevents proper movement in the channels of the chip. In such cases, shear a portion of the sample to reduce viscosity (see “Homogenization of HMW DNA Samples”, page 24). 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 for DNA. 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®).

Storage of HMW DNA

The elution buffer provided with the kit (10 mM Tris-HCl, pH 9.0, 0.5 mM EDTA) was 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.

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.

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 HEK293 and Human Blood Samples on the Oxford Nanopore Technologies Platform

	HEK293 SAMPLE 1	HEK293 SAMPLE 2	HUMAN BLOOD SAMPLE 1	HUMAN BLOOD SAMPLE 2
Mean read length	21338.9	19249.9	21522.6	24677.7
Mean read quality	12.8	13.2	13.4	13.3
Median read length	10388	9702	10130	12593
Median read quality	13.2	13.7	13.9	13.8
Number of reads	377687	633636	538090	327314
Read length N50	45432	40415	46542	51394
Total bases	8059414490 (8.1 Gb)	12197410796 (12.2 Gb)	11581090785 (11.6 Gb)	8077351338 (8.1 Gb)
Number, percentage and megabases of reads above quality cutoffs				
> Q5	377687 (100.0%) 8059.4 Mb	633636 (100.0%) 12197.4 Mb	538090 (100.0%) 11581.1 Mb	327314 (100.0%) 8077.4 Mb
> Q7	377685 (100.0%) 8059.3 Mb	633636 (100.0%) 12197.4 Mb	538090 (100.0%) 11581.1 Mb	327314 (100.0%) 8077.4 Mb
> Q10	346678 (91.8%) 7546.9 Mb	578938 (91.4%) 11333.7 Mb	498562 (92.7%) 10864.5 Mb	298205 (91.1%) 7496.1 Mb
> Q12	278174 (73.7%) 6218.2 Mb	484738 (76.5%) 9725.8 Mb	425604 (79.1%) 9438.5 Mb	253520 (77.5%) 6495.0 Mb
> Q15	22058 (5.8%) 333.2 Mb	109840 (17.3%) 1986.8 Mb	113362 (21.1%) 2348.0 Mb	60357 (18.4%) 1371.8 Mb
Top 5 highest mean basecall quality scores and their read lengths				
1:00	19.1 (332)	19.9 (263)	20.6 (156)	19.6 (332)
2:00	18.6 (268)	19.8 (142)	19.9 (214)	19.2 (1043)
3:00	18.5 (319)	19.8 (202)	19.5 (634)	19.2 (1317)
4:00	18.3 (645)	19.6 (631)	19.2 (366)	19.2 (570)
5:00	18.2 (1500)	19.2 (192)	19.1 (4073)	19.1 (303)
Top 5 longest reads and their mean basecall quality score				
1:00	441385 (13.0)	277445 (12.1)	298409 (12.3)	333983 (13.1)
2:00	331772 (12.8)	249785 (11.1)	282024 (13.3)	291973 (13.3)
3:00	299267 (13.2)	248331 (11.5)	280729 (14.1)	278424 (12.5)
4:00	292461 (13.1)	246374 (14.8)	275886 (12.5)	274430 (11.3)
5:00	272042 (13.8)	244162 (13.5)	271731 (12.1)	273123 (12.2)

DNA used for the sequencing libraries was extracted by following the standard protocols for cultured cells and fresh mammalian blood samples, without further size selection. 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, or shorter if no more data was generated by the flow cell. No additional treatment of the flow cell (e.g., flushing) was employed. In the samples that resulted in > 10 Gb of data, 800–1,000 ng of DNA library was loaded onto the flow cell for optimal sequencing performance and effective pore usage. Read lengths are indicated in bases.

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 Yield

- DNA did not attach to the 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 (≥ 5 x 10^6 cells or ≥ 1 ml blood), especially when low agitation speeds are used for UHMW DNA, additional inversions are required for the DNA to bind completely and tightly enough to the beads. Increase the binding time in the rotator to 8 minutes. Insufficient binding can lead to loss of DNA during the following short spin step (to dry the beads).

- Inaccurate quantitation of UHMW DNA concentration
  - Measuring 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“ on page 25 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”, page 24 to enable accurate quantitation.

Cells:

- Lysis volume too large
  - If working with ≤ 5 x 10^5 cells in the standard lysis volume, the efficiency of binding to the glass beads will be reduced as the DNA concentration is too low for efficient attachment. In such cases, a 3X reduction of the sample volume is needed. Use of the "low input" protocol will establish optimal binding conditions and improve yields.

- Inaccurate cell count
  - Some types cultured cells (e.g., HEK293) tend to clump and may be difficult to accurately count. Clumping usually leads to an underestimation of the cell count and an unexpectedly high yield. If the cell count of dilute samples is incorrectly estimated to be above 5 x 10^5, the use of standard input protocols may lead to reduced efficiency of the prep and lower yield.

- Cells lost in pelleting step at supernatant removal
  - While spinning down the cells, be sure to note which side of the tube is pointing outside in the centrifuge, to identify where the cell pellet is located. The cell pellet may not be visible by eye when working with low cell numbers. Keep the cell pellet side of the tube facing downward and remove the supernatant from the upper side of the tube. Leave < 20 µl of supernatant behind so that the pellet is not disturbed.

- Input amount too low
  - Working with < 1 x 10^5 cells is not recommended as recovery drops drastically below this number. Inputs in the range 1–5 x 10^5 cells require working with reduced buffer volumes for maximal recovery; follow "low input" protocol guidance.

Blood:

- Low input, small lysis volume needed
  - If working with < 500 µl blood (minimum is 100 µl) in the standard lysis volume, the efficiency of binding to the glass beads will be reduced as the DNA concentration is too low for efficient attachment. In such cases a 3X reduction of the sample volume is needed. Use the "low input" protocol to establish optimal binding conditions and improve yields.

- Leukocytes lost with supernatant removal during erythrocyte lysis
  - After spinning down the leukocytes during the erythrocyte procedure, be sure to note which side of the tube is pointing outside in the centrifuge, to identify where the leukocyte pellet is located. The pellet may not be clearly visible by eye when working with low input amounts or certain frozen blood samples. Keep the pellet side of the tube facing downward and remove supernatant from the upper side of the tube. Leave < 20 µl of supernatant behind so that the pellet is not disturbed.

- Blood was thawed before adding RBC Lysis Buffer
  - Thawing blood samples before adding RBC Lysis Buffer will result in nuclease activity, leading to a significant reduction in DNA size and yield. Keep frozen blood samples frozen, add cold RBC Lysis Buffer directly to the frozen samples, and carefully follow the guidance in the protocol for handling frozen blood samples.

- Blood sample is too old
  - Fresh (unfrozen) whole blood should not be older than a week. Older samples will show a progressive amount of DNA degradation and loss of yield.

- Excess supernatant left behind after erythrocyte lysis
  - If there is excess protein in the lysate prior to the addition of isopropanol for the precipitation step, the contaminating protein inhibits proper binding of the DNA to the beads. The precipitated DNA will float within the lysate and cannot bind to the beads efficiently. Additionally, the glass beads will tend to get stuck in the bottom of the 2 ml tubes. It is therefore very important to remove as much of the hemoglobin-containing supernatant as possible from the erythrocyte pellet during a blood prep; do not leave more than 20 µl behind.

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 long term storage at 4°C.

- 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 negatively. DNA samples purified using maximum agitation speeds have shorter fragment lengths; these can be pipetted with regular pipette tips and can be vortexed briefly.

Blood:

- Blood sample is too old
    - Fresh (unfrozen) whole blood should not be older than a week. Older samples will show a progressive amount of DNA degradation and loss of yield.

- Blood sample was thawed before adding RBC Lysis Buffer
    - Thawing blood samples before adding RBC Lysis Buffer will result in nuclease activity, leading to a significant reduction in DNA size and yield. Keep frozen blood samples frozen, add cold RBC Lysis Buffer directly to the frozen samples, and carefully follow the guidance in the protocol for handling frozen blood samples.

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 [1×10^7 for cells at max agitation speed, 5×10^6 cells for low agitation speeds, or 2 ml blood], 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 (4 minutes in vertical rotating mixer or 8 minutes when working with high input amounts), as this will produce excessive compacting and tangling of the HMW DNA and lead to inefficient resuspension of the DNA during elution.

- Incomplete resuspension or cell pellet at nuclei preparation step
    - Clumped cells that are not completely resuspended during the nuclei preparation steps may result in tangled DNA aggregates that cannot be dissolved completely after elution. Pay special attention to complete resuspension and lysis of the cells during the nuclei preparation step to obtain best results.

Blood:

- Leukocyte pellets not completely resuspended
  - When working with frozen blood or blood that is several days old, leukocytes tend to be sticky and difficult to resuspend. Clumped cells that are not completely resuspended during the leukocyte pelleting steps and the nuclei preparation step may result in DNA aggregates that cannot be dissolved completely after elution. Ensure cells are completely resuspended after all pelleting steps to obtain best results.

- Too much time taken for the erythrocyte lysis
  - Proceed through the erythrocyte lysis as rapidly as possible. Extended incubation with the RBC Lysis Buffer reduces leukocyte viability, causing stickiness and difficulty in resuspension.

Protein Contamination

- Too much input material used, Proteinase K efficiency reduced
  - When sample input is exceeding the recommended amounts samples will be too viscous, resulting in reduced Proteinase K efficiency and incomplete protein removal.

- Undissolved DNA falsely interpreted as protein contamination
  - If eluted DNA has not completely dissolved, this will result in some turbidity of the solution, that may be detectable by eye and will also affect the A_{260}/A_{230} ratio of DNA samples. Upon complete dissolving of the DNA the A_{260}/A_{230} will go back to higher values that are typically > 2.0 if the DNA is clean.

Blood:

- Incomplete erythrocyte lysis
  - If the erythrocyte lysis steps are carried out too quickly, a substantial amount of hemoglobin will be carried over into the nuclei lysis (which is when the Proteinase K is added). Due to the larger amount of hemoglobin, the Proteinase K activity present may not be sufficient to degrade all of it, resulting in protein contamination. Follow the guidance on erythrocyte lysis times carefully to avoid protein contamination.

- Too much supernatant left on leukocyte pellet
  - If too much supernatant is left on the leukocyte pellet during the erythrocyte lysis steps, a substantial amount of hemoglobin will be carried over into the nuclei lysis (which is when the Proteinase K is added). The amount of Proteinase K used in the nuclei lysis will be insufficient to handle that level of protein, resulting in protein contamination. Follow the detailed guidance on supernatant removal to avoid protein contamination.

- Sample is very erythrocyte-rich (e.g., rabbit blood)
  - The blood of some animal species, like rabbit, is extremely rich in erythrocytes and these erythrocytes are not fully depleted during lysis. As a result, there will be significant carryover of protein into the nuclei lysis, and the capacity of the Proteinase K used during that step will be overwhelmed. Work with reduced input amount (200 µl instead of 500 µl) to ensure complete protein removal.

RNA Contamination

- RNase incubation step omitted in cell protocol
  - Cultured cells typically have high levels of RNA. To allow for complete RNA removal, the protocol includes a 2-step lysis procedure in which cell walls are lysed while nuclei are kept intact to keep the viscosity low in the lysate. This allows optimal performance of the RNase A to degrade the RNA that is released from the cytosol. Be sure to incubate samples with RNase A for at least 2 minutes at room temperature. Incubation is not necessary for leukocytes, as their RNA content is low following the erythrocyte lysis procedure.

Ratio A260/A230 > 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.
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Ordering Information

PRODUCT	NEB #	SIZE
Monarch HMW DNA Extraction Kit for Cells & Blood	T3050S/L	5/50 preps
Monarch DNA Capture Beads	T3005L	200 Beads
Monarch Spin Collection Tubes	T2118L	100 collection tubes
Monarch Bead Retainers	T3004L	100 Retainers
Monarch gDNA Nuclei Prep & Lysis Buffer Pack	T3059L	1 pack; 20 ml of each buffer
Monarch RBC Lysis Buffer	T3051L	160 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	Updated protocol text. Updated DNA storage text in final step of the protocol	1/21
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.