Monarch® HMW DNA Extraction Kit: Blood Protocol

NEB #T3050

We strongly recommend that first-time users read the product manual at www.neb.com/T3050 and review the entire protocol before beginning. The product manual provides additional commentary and considerations for various steps. This shortened protocol is meant for experienced users. Access the manual, protocols and other resources using the QR code to the right.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Microcentrifuge
- Thermal mixer containing a 2 ml block (1.5 ml block can also be used)
- Ice or cooling block
- Ethanol (≥ 95%)
- Cold 1X PBS
- Isopropanol
- 1.5 ml microfuge tubes (DNase-free, DNA low bind recommended)
- Vertical rotating mixer
- Wide-bore pipette tips

IMPORTANT NOTES BEFORE YOU BEGIN

- ❑ Store RNase A and Proteinase K at -20°C and RBC Lysis Buffer at 4°C upon opening the kit.
- ❑ Add ethanol (≥ 95%) to the gDNA Wash Buffer concentrate as indicated on the bottle label.
- ❑ Cool PBS, RBC Lysis Buffer and Nuclei Prep Buffer to 4°C; store Nuclei Prep Buffer at 4°C for convenience.
- ❑ Set centrifuge to 4°C.
- ❑ Preheat thermal mixer with 2 ml block to 56°C.
- ❑ See product manual for validated sample types, guidance on working with volumes > 500 µl, and instructions for processing nucleated blood samples.

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INPUT GUIDANCE		
BLOOD SOURCE	PROTOCOL DESIGNATION	INPUT AMOUNT
Mammalian (non-nucleated)	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

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.**
2. **Incubate on ice; when sample starts to turn translucent (6-12 minutes for fresh blood, 5-7 minutes 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.** Process samples quickly to avoid clumping.
3. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then remove most of the supernatant by pipetting, leaving ~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 and add 1.5 ml of cold RBC Lysis Buffer. Vortex to resuspend completely. Incubate on ice for 3 minutes.**
5. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then carefully remove most of the supernatant by pipetting, leaving ~15-20 µl behind to avoid disturbing the pellet as described in Step 3. Close the cap.**
6. **Flick the tube or drag along a microtube rack to resuspend the pellet. Briefly vortex and add 1.5 ml of cold PBS. Vortex to mix.**
7. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C and remove supernatant by pipetting, leaving behind ~15-20 µl as described in Step 3. Close the cap and proceed immediately to Part 2: Leukocyte Lysis.**

❑ Frozen Mammalian Blood

Review important guidance in the Product Manual before working with frozen blood samples.

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.**
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.** Remove from rotator as soon as sample is thawed, as extended exposure to warm temperatures will harm the leukocytes.
4. **After frozen blood has thawed completely, vortex to resuspend any cell aggregates and proceed immediately to Step 5, keeping samples 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 ~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.
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.
7. **Pellet leukocytes for 3 minutes at 1,000 x *g* at 4°C, then remove supernatant by pipetting, leaving behind ~15-20 µl as described in Step 5. Close the cap and proceed immediately to Part 2:**

Leukocyte Lysis. If working with rabbit blood, repeat the PBS wash to reduce hemoglobin content.

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 to the next sample. Avoid introducing air bubbles.**

   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 at least 10 times (20 times for frozen samples) to resuspend completely.** The sample will become less turbid, indicating cell lysis; nuclei remain intact. If nuclei solutions 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 standard ligation-based nanopore sequencing, 2,000 rpm is recommended. For the largest DNA, use 300 rpm. Optimization may be required depending on the quality of the starting sample. Refer to “Choosing Agitation Speed During Lysis” in the product manual for guidance. If desired, samples can be stored at 4°C overnight after this incubation.

4. **If working with multiple samples, prepare and label the plastics for Part 3.** Each sample will require (1) Monarch Collection Tube II, (1) Monarch Bead Retainer, (1) Monarch 2 ml Tube, (1) 1.5 ml microfuge tube (DNA low bind recommended, not provided).

5. **Add 75 µl (Low Input: 25 µl) Precipitation Enhancer after the 10-minute incubation and mix by inverting 8-10 times. Proceed to Step 1 of Part 3.**

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**

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agitation speeds were used during lysis. If a vertical rotating mixer is not available, invert slowly and gently by hand 25-30 times. A manual inversion is complete when the tube returns to the upright position. Slow inversion is critical for the DNA to bind to the beads; each full inversion should take ~5-6 seconds.

3. **Remove liquid by pipetting. Avoid removing any of the gDNA wrapped around the glass beads.** Keeping tube upright, insert pipette tip and gently push beads aside to remove liquid, or tilt the tube almost horizontally and remove liquid from the top of the angled tube.
4. **Add 500 µl gDNA Wash Buffer, close the cap and mix by inverting the tube 2-3 times. Remove the wash buffer as described in the previous step.**
5. **Repeat the wash in Step 4, and remove the wash buffer by pipetting.** Alternatively, remove the buffer by carefully decanting. It is not necessary to remove all of the buffer at this point.
6. **Place a labeled bead retainer into a Monarch Collection Tube II. Pour the beads into the bead retainer and close the cap. Discard the used Monarch 2 ml Tube.** If working with multiple samples, be sure to close the cap after each bead transfer.
7. **Pulse spin (≤ 1 second) the sample in a benchtop minicentrifuge to remove 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 a labeled 1.5 ml microfuge tube (DNA low bind recommended, not provided). Discard the used collection tube.**
9. **Add 100 µl Elution Buffer II onto the glass beads and incubate for 5 minutes at 56°C in a thermal mixer with agitation at 300 rpm. Halfway through the incubation, ensure beads are not stuck by tilting the tube almost horizontally and gently shaking; do not let liquid reach the cap, and avoid splashing.**
10. **Ensure bead retainer is inserted into the 1.5 ml microfuge tube. Pour eluate and beads into the bead retainer and close the cap.**
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.** See product manual for guidance on preparing DNA for downstream use. Samples can be stored at 4°C for future use. The elution buffer (10 mM Tris, pH 9.0, 0.5 mM EDTA) is formulated for long term storage of gDNA.

Questions? Our tech support scientists would be happy to help. Email us at **info@neb.com**

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