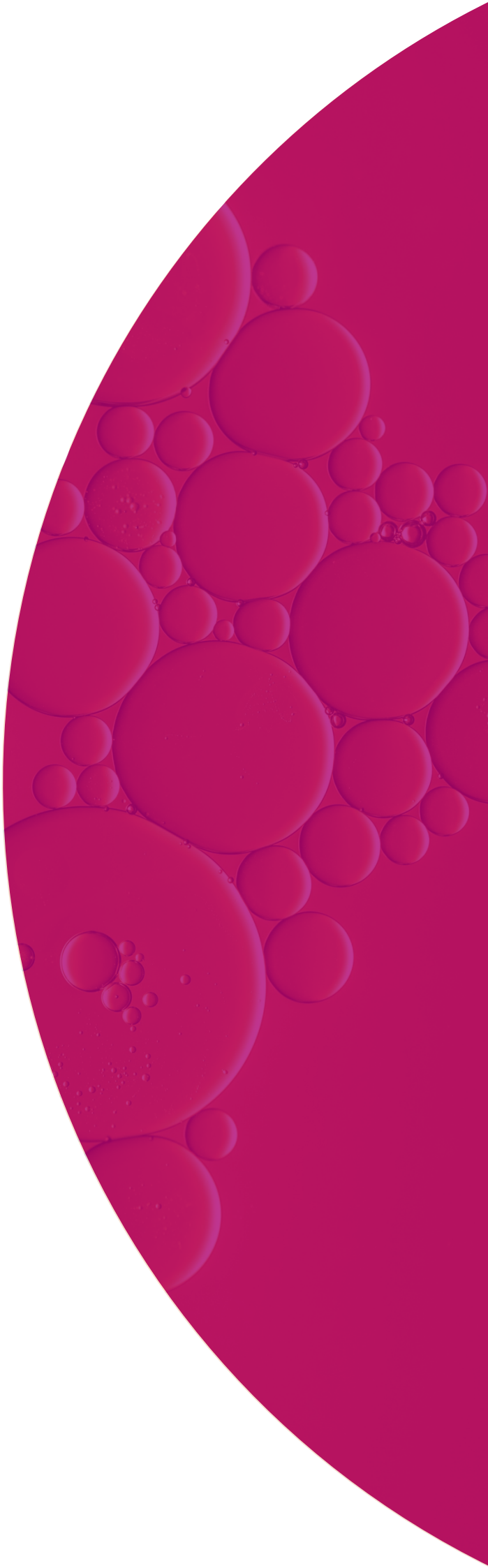
PacBio

Extracting HMW DNA using the Nanobind® HT CBB kit for nucleated red blood cells (nRBCs) on the Hamilton NIMBUS Presto system

Procedure & checklist



This procedure describes the workflow for high-throughput automated extraction of HMW (50–300 kb) DNA from 5 µL of nucleated red blood cells (nRBCs) using the Hamilton NIMBUS Presto robotic instrument. This procedure requires the Nanobind HT CBB kit (102-762-700) and is recommended for HiFi sequencing.

The Nanobind HT CBB kit has enough reagents for 96 extractions to be run in one of the following formats: 1 run x 96 samples, 2 runs x 48 samples, or 4 runs x 24 samples. We do not recommend running fewer than 24 samples per run as the kit is designed to accommodate dead volumes for a maximum of 4 runs (4 runs x 24 samples).

Required equipment and materials

Equipment/reagent	Manufacturer (part number)
Nanobind® HT CBB kit	PacBio® (102-762-700)
NIMBUS Presto assay ready workstation	Hamilton Company
KingFisher Presto 96 deep-well head	Thermo Fisher Scientific (24078830)
KingFisher 96 deep-well plates	Thermo Fisher Scientific (95040450)
KingFisher 96 deep-well tip comb for deep-well magnets	Thermo Fisher Scientific (97002534)
60 mL Reagent Reservoir	Hamilton Company (56694-01)
200 mL Reagent Reservoir	Hamilton Company (56695-01)
1000 µL Conductive Filter Tips	Hamilton Company (235905)
300 µL Conductive Filter Tips	Hamilton Company (235903)
300 µL Wide Bore 0.71 mm Orifice Conductive Filter Tips	Hamilton Company (235452)
Screw cap micro tube, 2 mL	Sarstedt Inc (72.694.406)
1x PBS	Any major lab supplier (MLS)
Ethanol (96–100%)	Any MLS
Isopropanol (100%)	Any MLS
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA Quantification	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits

Before you begin

Prior to starting

Buffer CW1 and CW2 are supplied as concentrates. This kit uses CW1 with a 60% final ethanol concentration. This kit uses CW2 with a 60% final ethanol concentration. Before using, add the appropriate amount of ethanol (96–100%) to Buffer CW1 and Buffer CW2 as indicated on the bottles.

Kit storage

RNase A should be stored at 4°C upon arrival.

Nanobind disks and all other buffers should be stored at room temperature (15–30°C).

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

Product use

Nanobind kits are intended for research use only.

Headspace

The automation script for the following protocol includes “headspace” volume in each plate. The “headspace” volumes are virtual volumes added to the automation script to improve the retention of the Nanobind disks on the magnetic rod and do not interfere with the extraction efficiency or performance.

Procedure and checklist

Input requirements

Amount: 2.5–20 µL of nucleated red blood cells (nRBCs)

- Nucleated red blood cells (nRBCs) are found in most vertebrate animals, with the exception of most mammals.
- Animals with nRBCs contain 10–100X more DNA per volume of blood compared to mammals.
- Use a volume of blood that will yield 5–20 µg DNA. This will vary from animal to animal.
    - Some tested inputs that yield 5–20 µg DNA are:
        - Chicken: 2.5 µL
        - Tuna: 5 µL
        - Vulture: 10 µL
        - Salmon: 20 µL
    - For initial testing, start with 5 µL to prevent overloading the chemistry and then adjust as necessary.
    - Warning: Input volumes that are too high may result in Nanobind disks being "dropped" in the Lysis/Binding solution and/or cause well-to-well contamination.
- Blood should be frozen as quickly as possible after being drawn in small aliquots to avoid repeated freeze-thaws.
- Storage at 4°C should be limited to 2 days or fewer to prevent sample degradation.
- K2 EDTA is the recommended anticoagulant. Samples stored in sodium heparin (NaHep) and citrate (NaCit) also performed well in limited testing.
- This protocol has been validated on nRBCs from Pacific bluefin tuna (*T. orientalis*) and chicken (*G. gallus*).
- No systematic difference has been observed in DNA QC or sequencing results between fresh and frozen nRBCs.
- nRBCs preserved in >70% ethanol performed well in limited testing. Use the equivalent of 5 µL of blood.
    - For example, the volume equivalent of 5 µL of unpreserved nRBCs is ~17 µL if the nRBCs are preserved with 70% ethanol or 50 µL if the nRBCs are preserved with 90% ethanol.

Prior to beginning protocol

- If using frozen nRBCs, incubate in a water bath or dry block heater at 37 °C for 15 minutes to thoroughly thaw the sample. Mix the sample by inverting the tube >15 times immediately prior to use. **Improperly thawed and mixed samples may result in inconsistent DNA yield and purity.**
- Work with Hamilton to ensure the NIMBUS Presto assay ready workstation is properly configured and has the correct methods installed.
- Prepare an .xls worklist based on the example below. Column 1 should begin with “Sample_ID” and list one sample ID for each sample being processed, from “Sample1” up to “Sample 96”. Column 2 should begin with “Sample_Position” and list out the corresponding well of the sample being processed, from “A1” up to “H12”. If processing fewer than 96 samples, leave all rows blank underneath the last sample ID and sample position.

	A	B	C	D
1	Sample_ID	Sample_Position		
2	Sample1	A1		
3	Sample2	B1		
4	Sample3	C1		
5	Sample4	D1		
6	Sample5	E1		
7	Sample6	F1		
8	Sample7	G1		
9	Sample8	H1		
10	Sample9	A2		
11	Sample10	B2		
12	Sample11	C2		
13	Sample12	D2		
14	Sample13	E2		
15	Sample14	F2		
16	Sample15	G2		
17	Sample16	H2		
18	Sample17	A3		
19	Sample18	B3		
20	Sample19	C3		
21	Sample20	D3		
22	Sample21	E3		
23	Sample22	F3		
24	Sample23	G3		
25	Sample24	H3		
26				
27				

HMW DNA extraction – nucleated red blood cells (nRBCs)

This procedure describes the workflow for automated HMW DNA extraction from 5 µL of nucleated RBC blood on the Hamilton NIMBUS Presto robotic instrument. This protocol uses the KingFisher 96 deep-well magnetic head, 96 deep-well plates, and 96 deep-well tip comb. This protocol cannot be run with the 24 deep-well head and 24 deep-well plates.

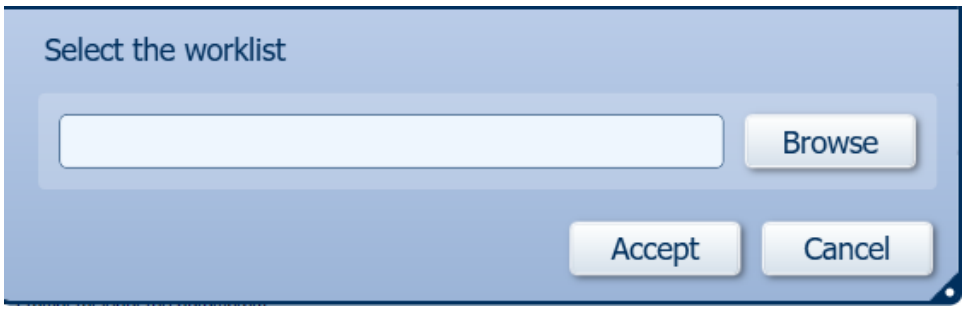
1. Collect 3 KingFisher 96 deep-well plates and prepare as indicated in the following table. **Add the components to Plate 1 (Lysis/Binding) only after all other plates have been prepared.**

Plate Number	Plate Name	Reagent	Volume per well
1	Sample Plate	Sample + reagents from Step 2	
2	Nanobind Storage Plate	One 3 mm Nanobind Disk per well*	
3	Tip Plate	KingFisher 96 deep-well tip comb	

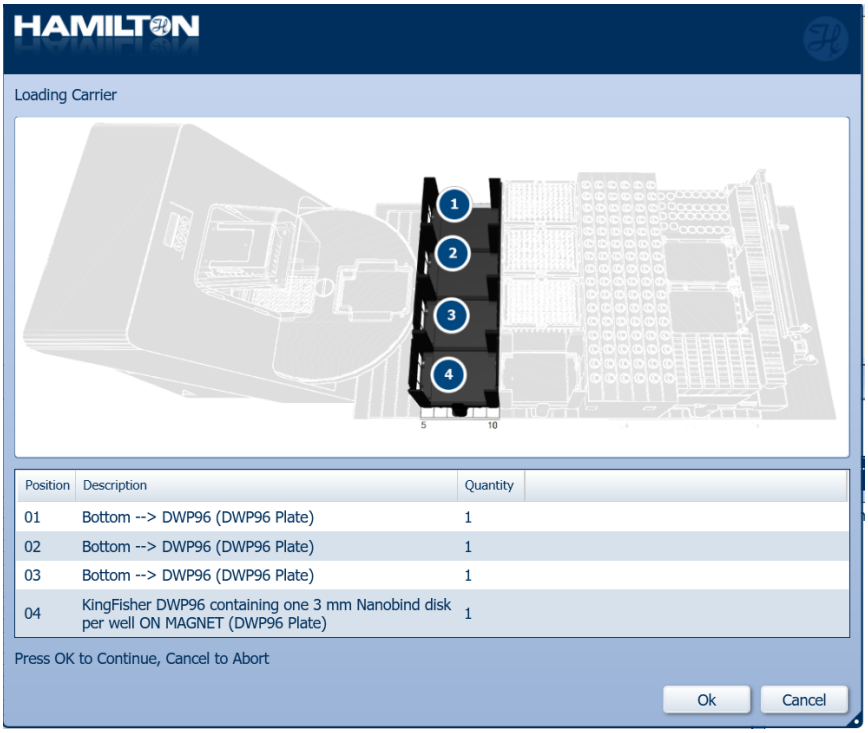
***Nanobind disks do not need to be perfectly centered in the wells, but ensure they are at the bottom of the well and not stuck to the side walls.**

2. Prepare sample in the Lysis/Binding Plate:
    - Add 195 µL of 1x PBS to each well.
        - **If using an input volume of nRBCs other than 5 µL, adjust the volume of 1x PBS accordingly so the total volume of nRBCs and PBS is 200 µL.**
    - Add 5 µL of nRBCs to each well.
        - If frozen, thaw nRBCs at 37°C using a water bath or dry block heater for 15 minutes. Inversion mix >15 times to thoroughly mix immediately prior to use.

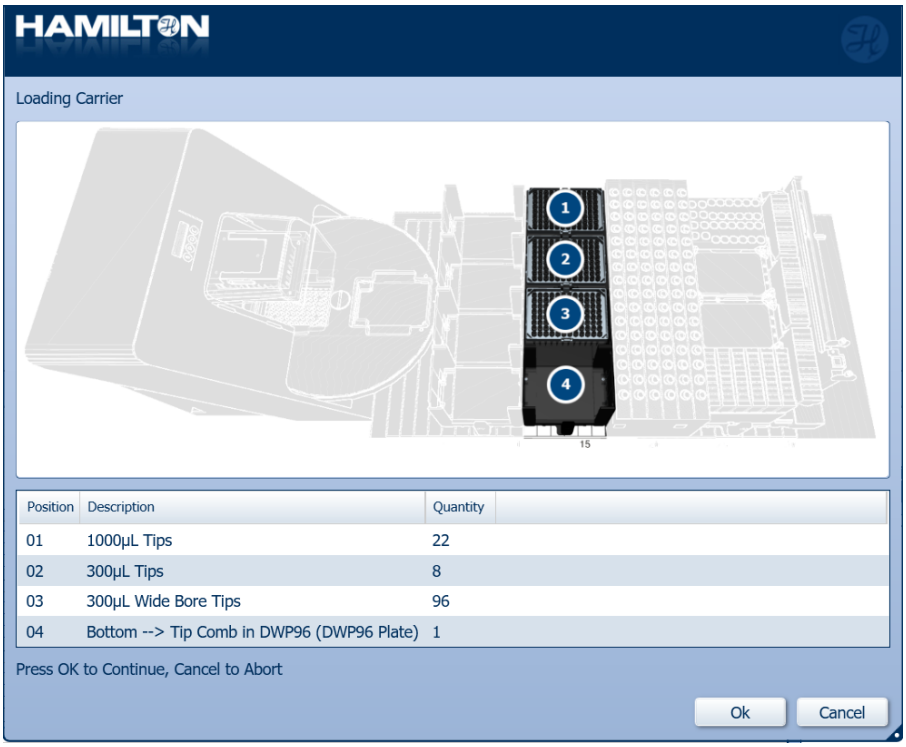
    Note: the sample and reagents MUST be added to the wells in the order described above.
3. Ensure the instrument is set up with the 96 deep-well magnetic head and the 96 deep-well heating block.
4. Select the **nRBC_Nanobind_HT_NIMBUS** script on the Hamilton NIMBUS Presto instrument computer. Follow the prompts to select the run parameters.
5. An .xls worklist is used to specify the plate coordinates of the samples (see **Prior to beginning protocol** section). Click “Browse” and select the appropriate worklist.



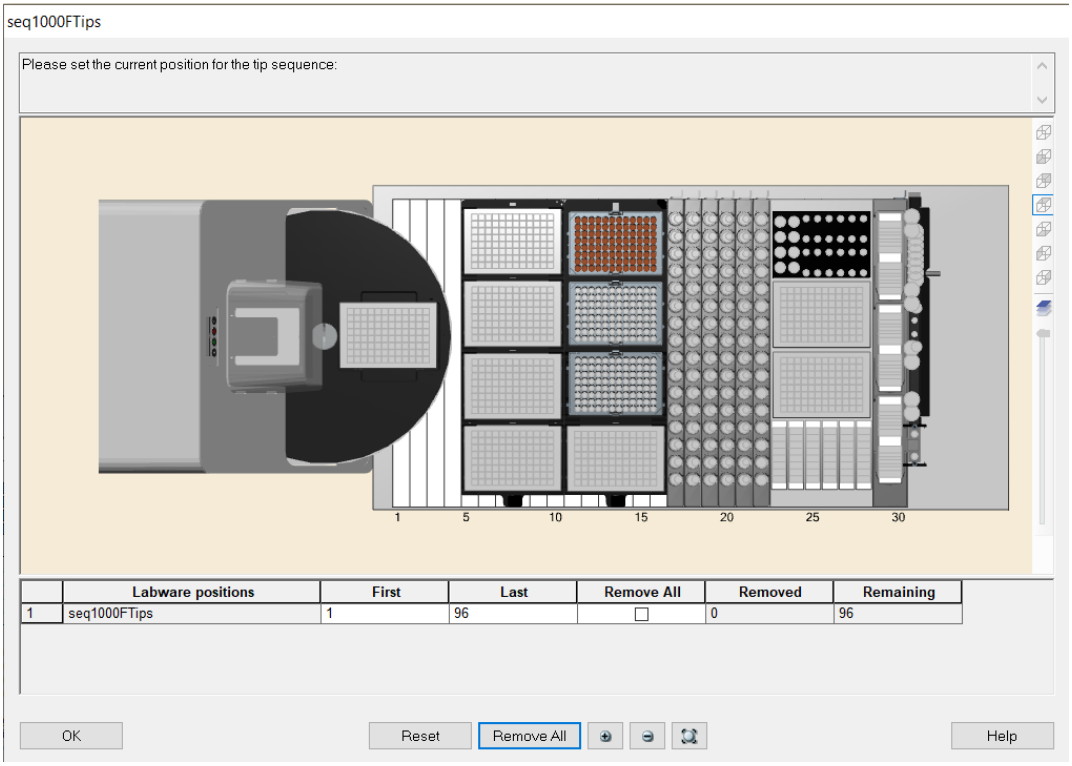
6. Load 3 empty KingFisher 96 deep-well plates on the list carrier positions. Load the Nanobind Storage Plate on top of the magnet on the listed carrier positions, then click “Ok”.



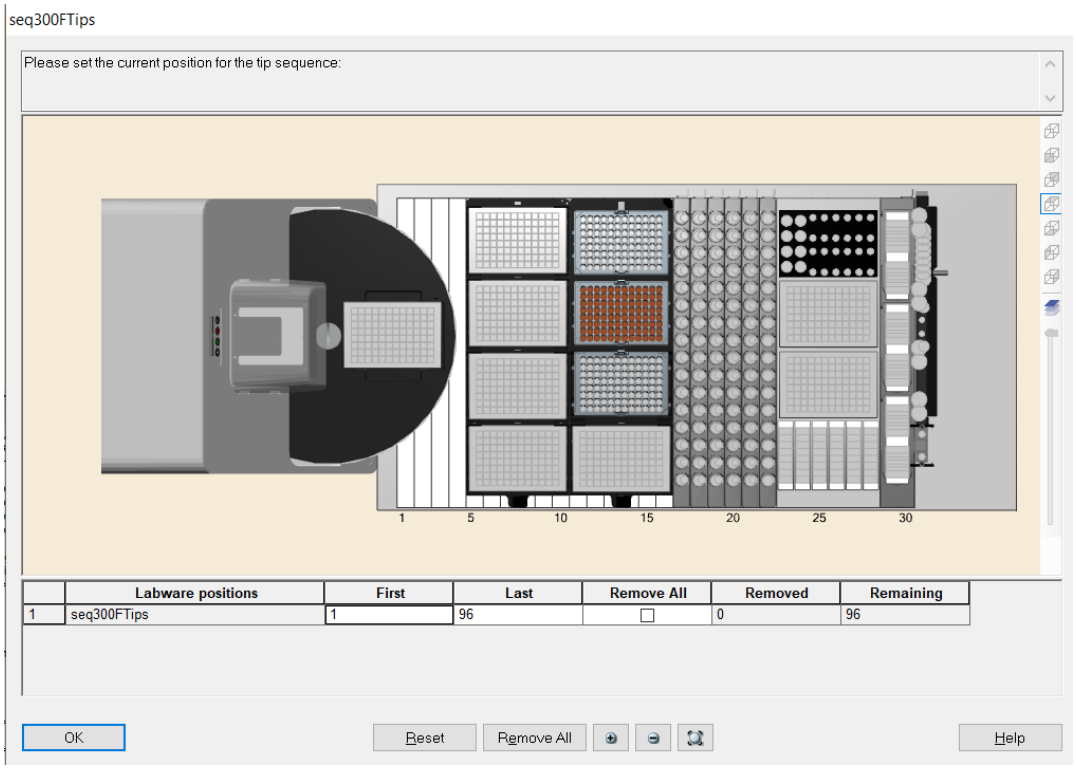
7. Load the listed number of 1000 µL conductive filter tips, 300 µL conductive filter tips, and 300 µL wide-bore conductive filter tips on the listed carrier positions. Load the Tip Comb Plate on the listed carrier position, then click “Ok”.



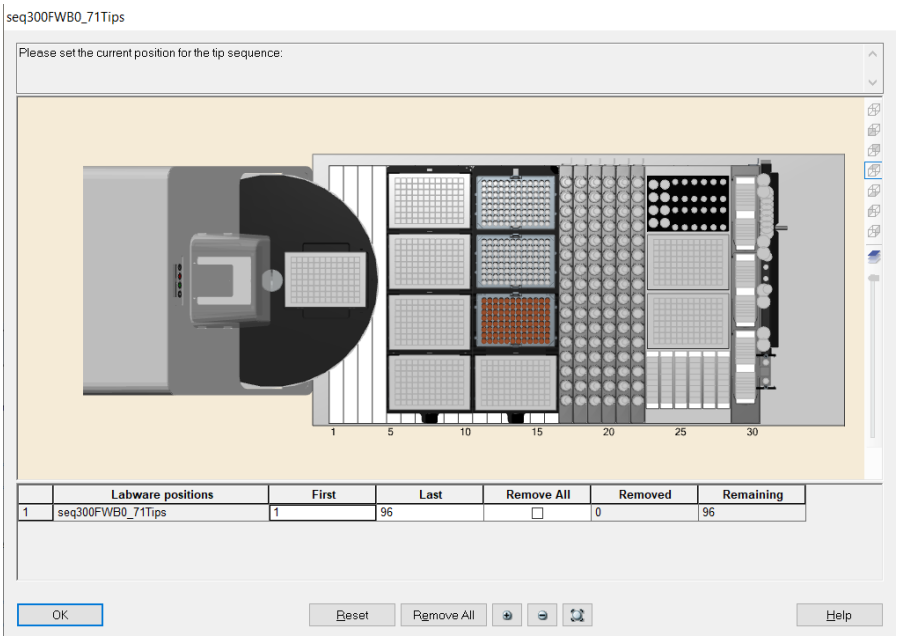
8. Use the cursor to set the first and last position of the 1000 µL conductive filter tips, then click “Ok”.



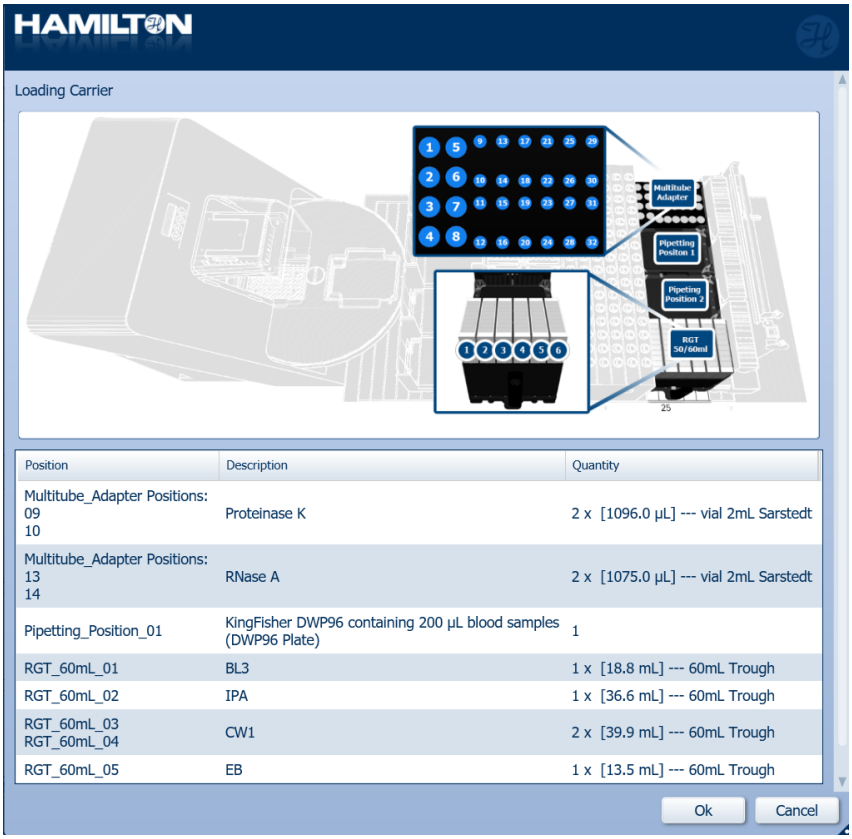
9. Use the cursor to set the first and last position of the 300 µL conductive filter tips, then click “Ok”.



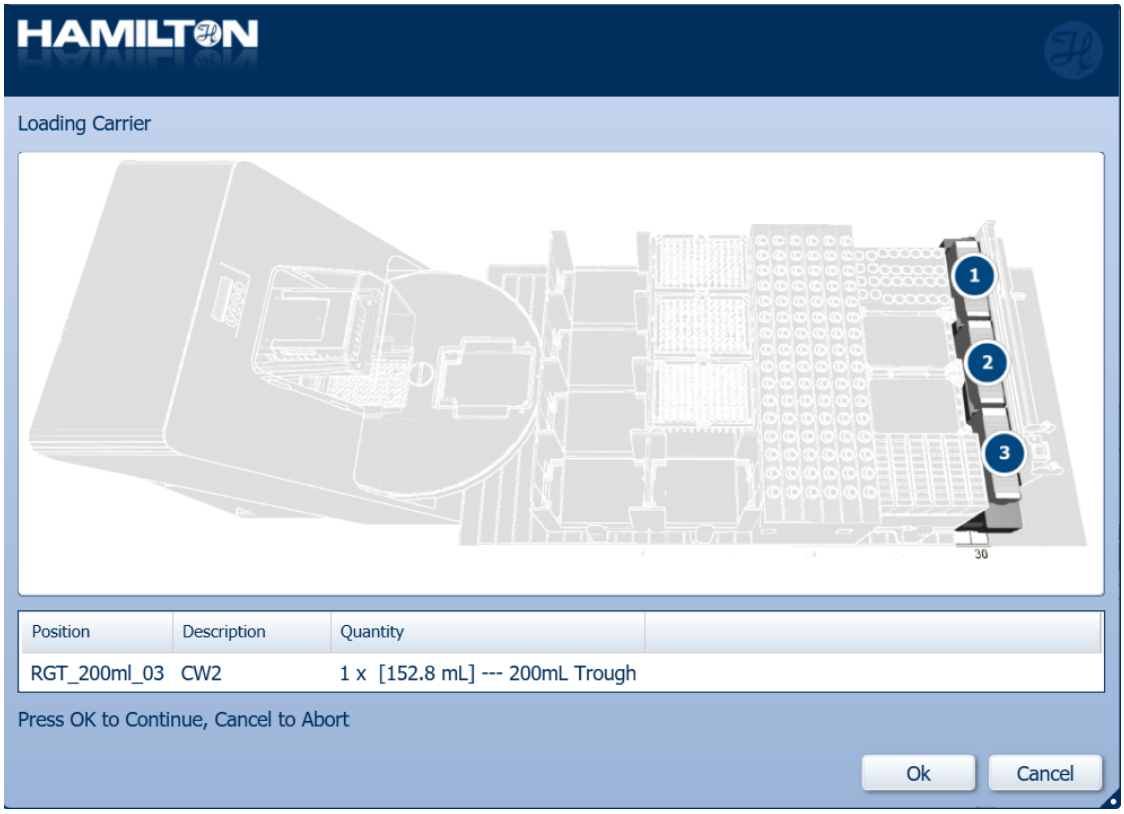
10. Use the cursor to set the first and last position of the 300 µL wide-bore conductive filter tips, then click “Ok”.



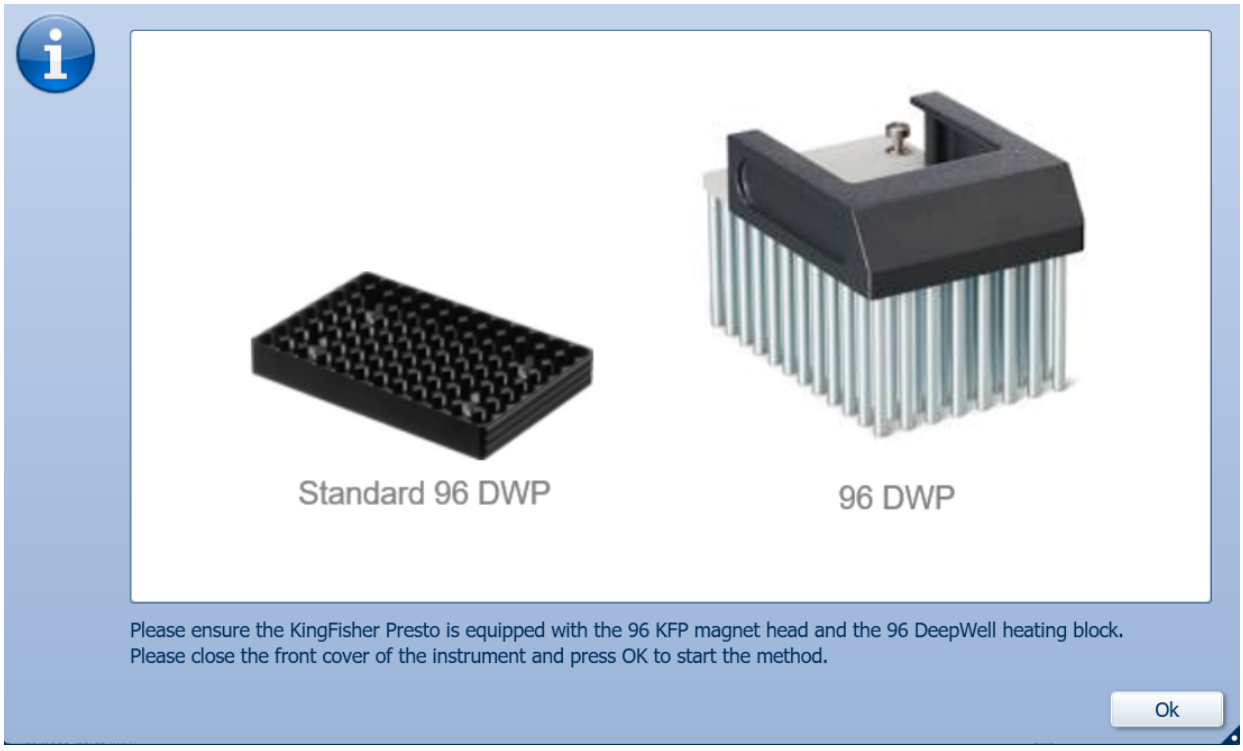
11. Fill the listed numbers of 2 mL screw cap micro tubes with the listed volumes of Proteinase K and RNase A, then load them on the listed positions in the MultiTube Adapter. Load the Sample Plate on the listed carrier position. Using a serological pipette, fill 60 mL reagent reservoirs with the listed volume of Buffer BL3, isopropanol, Buffer CW1, and Buffer EB and load them on the listed positions of the reagent reservoir carrier. Then, click “Ok”.



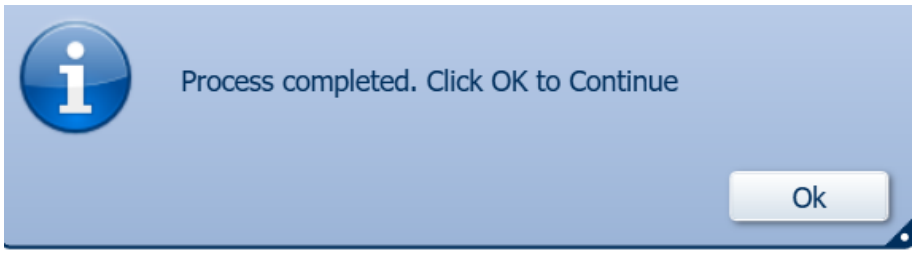
12. Fill one 200 mL reagent reservoir with the listed volume of Buffer CW2, then load it on the listed position of the reagent reservoir carrier. Then, click “Ok”.



13. Close the front cover of the instrument, then click “Ok” to start the method.



14. At the end of the run (~130 minutes after start), click “Ok” to end the run.



15. Remove plates from the instrument.
16. Transfer eluates from the Eluate Plate to a new storage plate or storage tubes if desired.
    - The protocol is designed to leave the Nanobind disks in the Elution Plate. On occasion, the disk may be transferred back to the tip comb storage plate after elution. This does not affect extraction performance.
17. Pipette mix the sample 10 times with a standard P200 pipette to homogenize and disrupt any unsolubilized “jellies” that may be present.
    - Take care to disrupt any regions that feel more viscous than other regions.
    - Limited pipette mixing will not noticeably reduce DNA size or sequencing read lengths but is important for accurate quantitation and consistent sequencing performance.
18. Let eluate rest overnight at RT to allow DNA to solubilize.
    - Visible “jellies” should disperse after resting.
19. Following overnight rest, pipette mix 10 times with a standard P200 pipette and analyze the recovery and purity as described in the **QC procedure** section.

QC procedure

It is recommended that QC is performed after the DNA has been allowed to rest at RT overnight and appears homogeneous under visual examination and when pipetting.

1. Perform a NanoDrop UV/VIS measurement to determine total nucleic acid concentration as well as purity (A260/A280, A260/A230).
    - If the DNA is very heterogeneous or contains large amounts of unsolubilized "jellies", refer to the **kit Guide & overview "Heterogeneity and viscosity"** section for more information.
2. Perform a Qubit dsDNA BR assay measurement to determine DNA concentration.
    - We recommend the Qubit 3.0 (Thermo Fisher Scientific) with the dsDNA BR assay kit. We do not recommend the dsDNA HS assay kit as we have found the concentration measurements to be unreliable.
3. Perform a Qubit RNA BR assay measurement to determine RNA concentration (optional).
    - We recommend the Qubit 3.0 (Thermo Fisher Scientific) with the RNA BR assay kit.
4. Use Agilent Femto Pulse for HMW DNA size QC.
    - We recommend diluting the sample to 250 pg/µL. Finger tap to mix.
    - Avoid mixing with a standard pipette. This will shear the DNA. Always use a wide-bore pipette when making dilutions.
    - Use the Genomic DNA 165 kb Kit (Agilent Technologies) for unsheared gDNA.

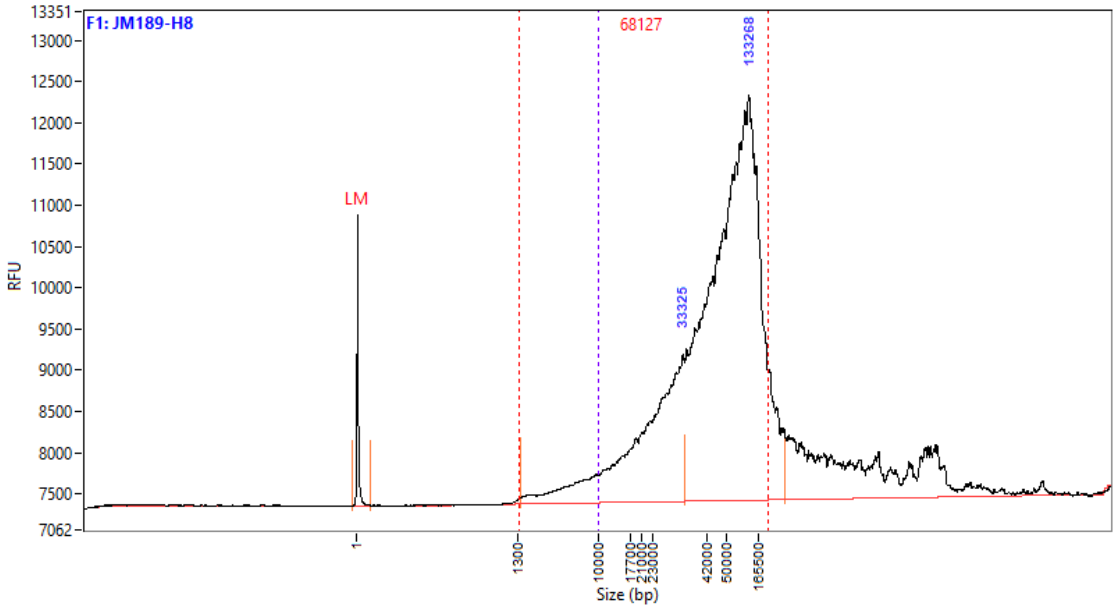
Storage of DNA

DNA can be stored in Buffer EB at 4°C for several months. Long term storage at −20°C or −80°C can be used if necessary. Avoid freeze/thaw cycles since this can degrade high molecular weight DNA.

Results

- DNA extracted from nRBCs will yield ~10–20 µg depending on donor hematocrit.
- 260/280 ratios should consistently be 1.8–2.0.
- 260/230 ratio can vary from 1.9–2.3.
- Samples with UV purities within the expected range should sequence well. UV purities outside of these ranges may indicate abnormalities in the extraction process.
- The mode of extracted nRBC DNA measured on the Femto Pulse system (Agilent Technologies) is typically 100 kb+.

Sample	Input	260/280	260/230	Nanodrop (ng/µL)	Qubit DNA yield (µg)
Pacific bluefin tuna	5 µL	1.86	2.26	151.5	13.00
Chicken	2.5 µL	1.86	2.17	136.2	11.40



DNA size distribution of unsheared gDNA isolated from a chicken sample using the Hamilton NIMBUS Presto on the Femto Pulse system (Agilent Technologies).

Troubleshooting FAQ

1. **What do I do if the DNA is heterogenous and/or contains visible insoluble "jellies"?**
   - HMW DNA is inherently difficult to work with. The longer the DNA, the more heterogeneous it will be.
   - Homogeneity can be improved by mixing 5–10 times with a standard P200 pipette. Take care to disrupt any particularly viscous regions. Overnight incubation at RT will then allow the HMW DNA to relax back into solution.

2. **I transferred the eluate, but there is still liquid or a gel-like material on the Nanobind disk. What do I do?**
   - Ensure all the DNA is recovered from the sample by visually inspecting the Nanobind disk after the eluate has been transferred. The Nanobind disk should appear mostly free of any substances. If any material remains on the Nanobind after elution, remove as much as possible using a P200 pipette. Leaving a small amount of DNA/liquid on the Nanobind disk should not have a large impact of DNA yield.
   - We do not recommend a second elution. This is usually unnecessary and will result in a diluted, less-concentrated DNA sample.

3. **Why is my DNA yield lower than expected?**
   - Ensure all the DNA was recovered from the Nanobind disk. See FAQ #2 for more information.
   - DNA yield can be affected by improper thawing and/or mixing of the blood immediately prior to beginning the Hamilton NIMBUS Presto protocol. Refer to the recommendations in the **Prior to beginning protocol** section to properly prepare the sample.
   - If the sample is heterogeneous, you may be sampling from an area of the eluate that is less concentrated. Take measurements from the top, middle, and bottom of the eluate to get an average concentration.
   - Occasionally, samples can yield lower DNA recoveries than expected. This is typically due to factors beyond the control of the protocol, such as inherent sample inhomogeneity from certain donors. If the DNA yield is insufficient for sequencing and additional blood sample remains, rerun the protocol and contact PacBio for further steps.

4. **Why are the purities lower than expected? Is this a problem?**
   - We do NOT see a correlation between UV purity and sequencing performance and do not pay particular attention to the UV purity if it is within the expected range for that particular sample type. Generally, nRBC DNA results in UV purities of 260/230 >1.9 and 260/280 >1.8. Samples with UV purity slightly outside of this range will likely still sequence well. Samples with UV purity far outside this range should be treated with caution.
   - DNA purity can be affected by improper thawing and/or mixing of the blood immediately prior to beginning the Hamilton NIMBUS Presto protocol. Refer to the recommendations in the **Prior to beginning protocol** section to properly prepare the sample.

5. **One or more of my eluates do not contain a Nanobind disk after completion of the protocol. What does this mean?**

- The Nanobind disks occasionally remain on the tip comb after elution and are returned to the tip comb storage plate at the end of the protocol. If the Nanobind corresponding to the sample in question has been returned to the tip comb storage plate, move forward with sample QC as this should not have significant effects on DNA recovery.
- If a Nanobind disk for the eluate in question is not on the tip comb in the tip comb storage plate, this sample will likely not contain any DNA. Occasionally, the disk can become dislodged from the magnet during binding and remains in the sample plate. This is a rare occurrence but can happen. We recommend rerunning the protocol if additional blood sample remains.
- If the Nanobind disk for the eluate in question is not in the tip comb storage plate and not in the sample plate, contact PacBio for further instruction.

6. **One or more of my eluates exhibit a slight red/orange tint. What does this mean?**

   - Occasionally some of the blood coloration carries through the extraction. This can result in a slight decrease in 260/230 purity, however it should not affect DNA yield or sequencing performance.

7. **Why isn't the protocol running and/or why is there an error message?**

   - Check to ensure the correct script is installed. See **Nanobind HT kit Guide & overview "Programs."**
   - Check to ensure the correct magnet head and heat block are installed in the KingFisher Presto.
   - Check to ensure all plates and reservoirs are in the correct positions. The KingFisher Presto will give an error message if it does not detect the tip comb (i.e., the tip comb is not in the correct position). The Hamilton NIMBUS Presto will give an error message if no liquid is detected or if there is insufficient volume.
   - For other on-instrument error messages, contact Hamilton.

Revision history (description)	Version	Date
Initial release	01	February 2024