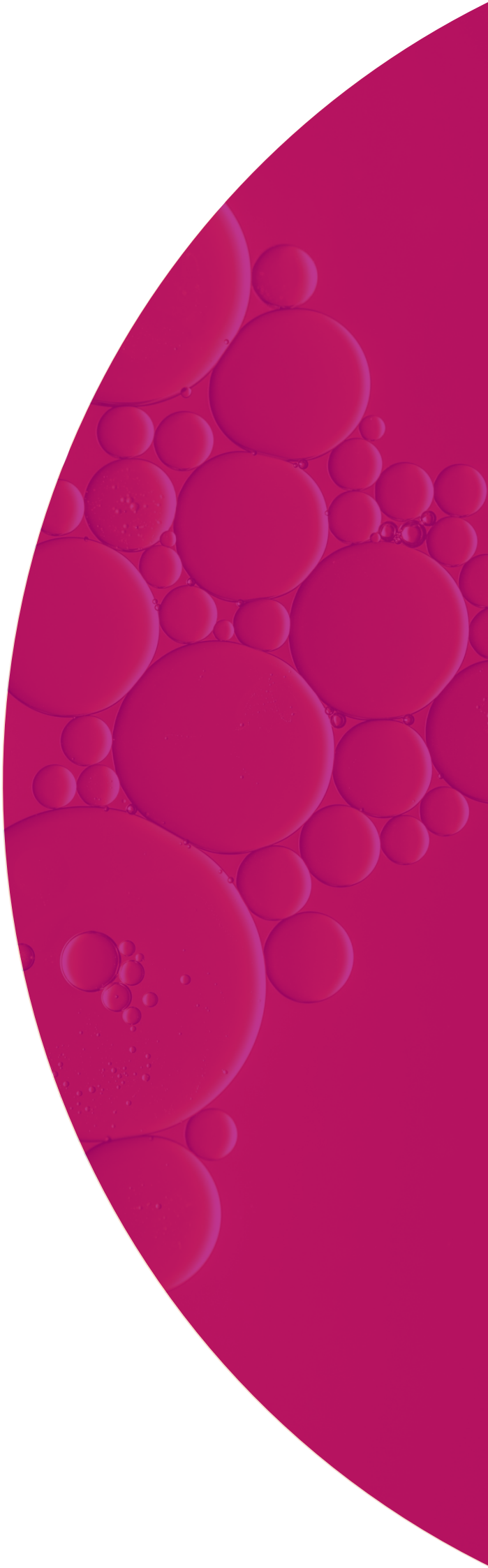
PacBio

Extracting HMW DNA using the Nanobind® HT CBB kit for cultured bacteria 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 cultured Gram-negative and Gram-positive bacteria using the Hamilton NIMBUS Presto robotic system. 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)
Tris-HCl, 1 M, pH 8.0	Invitrogen (15568025)
Ethylenediaminetetraacetic Acid (EDTA), 0.5 M, pH 8.0	Thermo Fisher Scientific (15575020)
Sucrose	Thermo Fisher Scientific (BP220)
Triton X-100	Sigma-Aldrich (X100)
Lysozyme	MP Biomedicals (100831)
Lysostaphin	Sigma-Aldrich (L7386)
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: 5x10^8 Gram-negative or Gram-positive bacteria

- *E. coli* generally have 5x10^9 cells per mL of a culture that is 1 OD600; however, different bacterial species may have different cell concentrations at the same OD600 measurement.
- Input should be scaled appropriately to have an amount of cells that will yield 5–25 µg of DNA.
    - For *E. coli* and *L. monocytogenes,* this was 0.5 mL of 1 OD600 culture.
    - Warning: cell inputs that yield >25 µg may result in Nanobind disks being "dropped" in the lysis/binding solution and/or cause well-to-well contamination.
- This protocol has been validated on Gram-negative bacteria including *E. coli, S. enterica, S. sonnei, K. pneumoniae,* and *P. aeruginosa*, and Gram-positive bacteria including *L. monocytogenes*, *E. faecalis*, and *S. aureus.*
- No systematic difference has been observed in DNA QC or sequencing results between fresh and frozen cells.

Prior to beginning protocol

- 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				

- Prepare the following buffer prior to beginning DNA extraction.

STET Buffer

Reagent	Final concentration
Tris-HCl	50 mM
EDTA	50 mM
Sucrose	8% (m/v)
Triton X-100	5% (v/v)
Lysozyme (added before use)	10 mg/mL

- STET buffer without lysozyme can be stored at 4°C. STET buffer + Lysozyme should be made fresh and once lysozyme is added, the buffer should be used the same day.
- After addition of lysozyme, incubate at 37°C for 10–15 minutes until lysozyme is fully dissolved.
- For some Gram-positive bacteria, such as *S. aureus*, lysostaphin should be used as a supplement. Add 2.5 µL lysostaphin to 50 µL STET buffer with lysozyme per extraction.
- For difficult-to-lyse bacteria, enzymatic cocktails may be necessary.

HMW DNA extraction – cultured Gram-negative and Gram-positive bacteria

This procedure describes the workflow for automated HMW DNA extraction from cultured Gram-negative and Gram-positive bacteria 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.

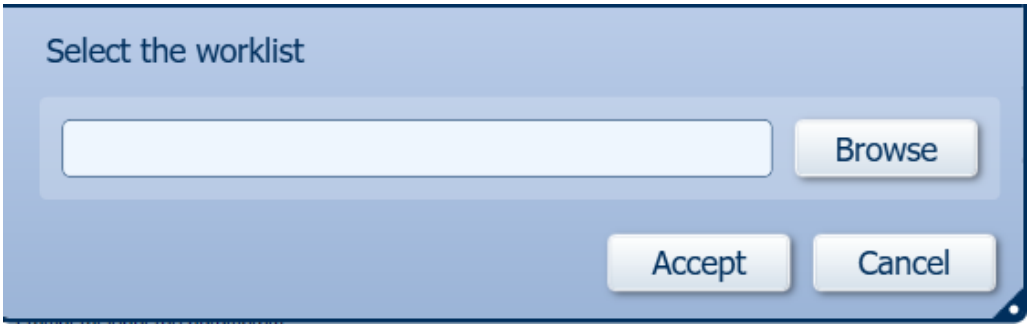
1. Collect 3 KingFisher 96 deep-well plates and prepare as indicated in the following table. **Add the components to Plate 1 (Sample Plate) only after all other plates have been prepared.**

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	

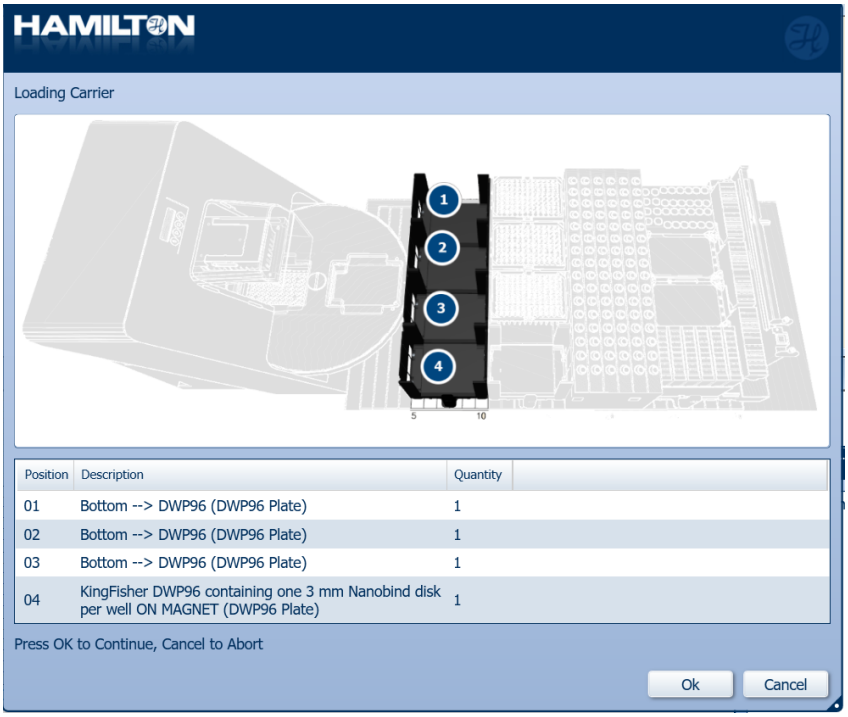
   ***Nanobind disks do not need to be perfectly centered in the wells, but ensure they are at the bottom of the well and not stuck to the side walls.**

2. Prepare sample: Harvest cells and centrifuge at 16,000 x *g* for 1 minute at 4°C to pellet cells in a 1.5 mL Protein LoBind tube; remove the supernatant.
   - Frozen cell pellets may be substituted here.
   - Use $5x10^8$ cells or 0.5 mL of a culture that is 1 OD600.
   - Input should be scaled appropriately to have an amount of cells that will yield 5-25 µg of DNA.
   - **Warning: cell inputs that yield >25 µg may result in Nanobind disks being "dropped" in the Lysis/Binding solution and/or cause well-to-well contamination.**
3. Add 10 µL of 1x PBS and pipette mix 10 times with a standard P200 pipette to resuspend cells.
   - Mix until cell pellet is fully resuspended without visible lumps. Sticky cell types may require additional pipette mixing or vortexing.
   - Additional mixing at this step will not affect DNA size. However, incomplete resuspension will result in inefficient lysis and digestion which will lead to low yield, low purity, and high heterogeneity.
4. Add 50 µL of STET Buffer + lysozyme and pulse vortex for 1s x 10 times (max setting).
   - For some Gram-positive bacteria, such as *S. aureus*, lysostaphin should be used as a supplement. Add 2.5 µL lysostaphin to 50 µL STET buffer + lysozyme per extraction.
5. Prepare sample in the Sample Plate (Plate 1):
   - Add the samples prepared in Steps 2-4 to individual wells.
6. Ensure the instrument is set up with the 96 deep-well magnetic head and the 96 deep-well heating block.
7. Select the **Bacteria_Nanobind_HT_NIMBUS** script on the Hamilton NIMBUS Presto instrument computer. Follow the prompts to select the run parameters.

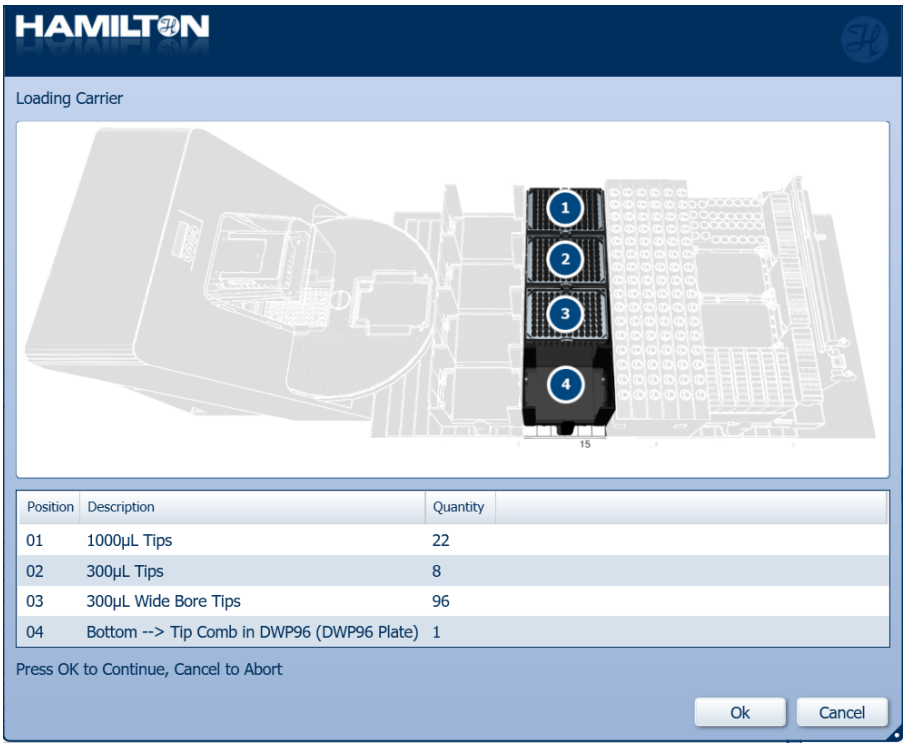
8. An .xls worklist is used to specify the plate coordinates of the samples (see **Prior to beginning protocol** section). Click “Browse” and select the appropriate worklist.



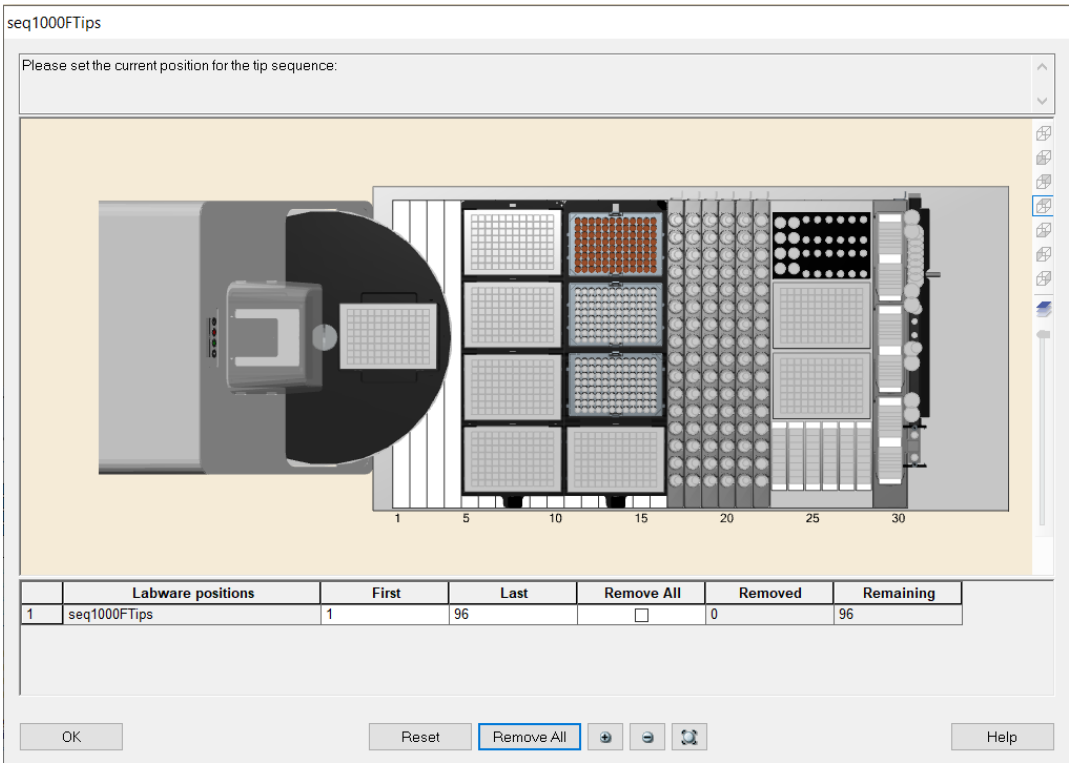
9. Load 3 empty KingFisher 96 deep-well plates on the list carrier positions. Load the Nanobind Storage Plate on top of the magnet on the listed carrier positions, then click “Ok”.



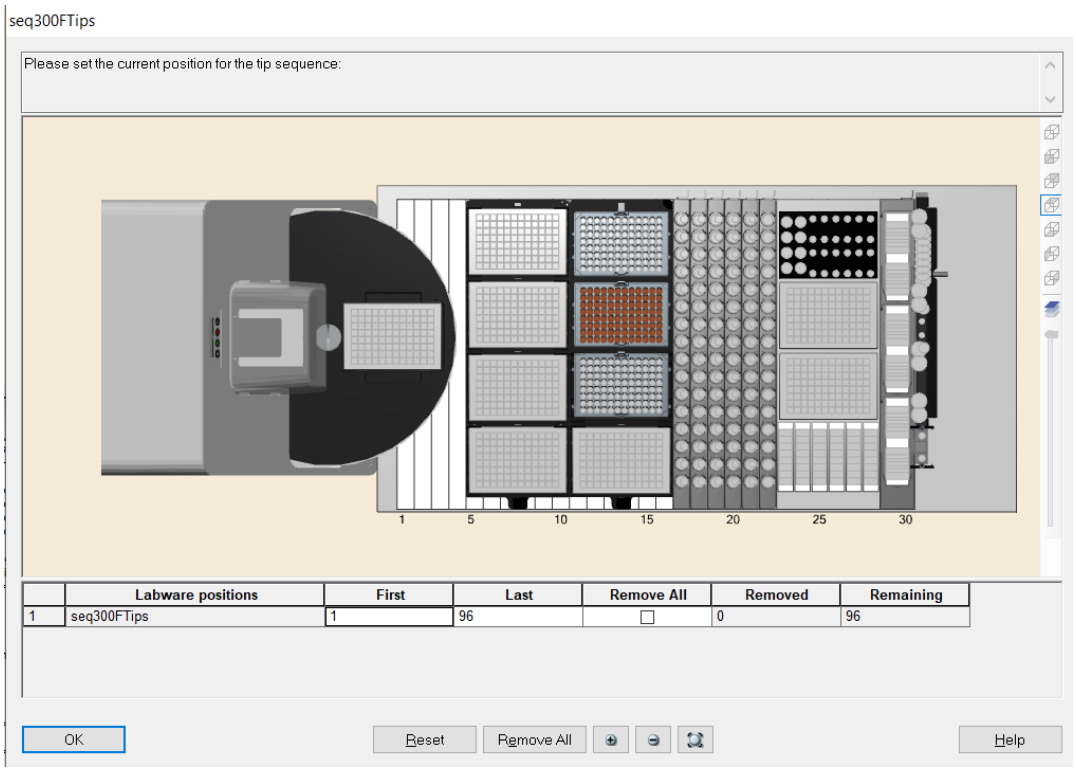
10. Load the listed number of 1000 µL conductive filter tips, 300 µL conductive filter tips, and 300 µL wide-bore conductive filter tips on the listed carrier positions. Load the Tip Comb Plate on the listed carrier position, then click “Ok”.



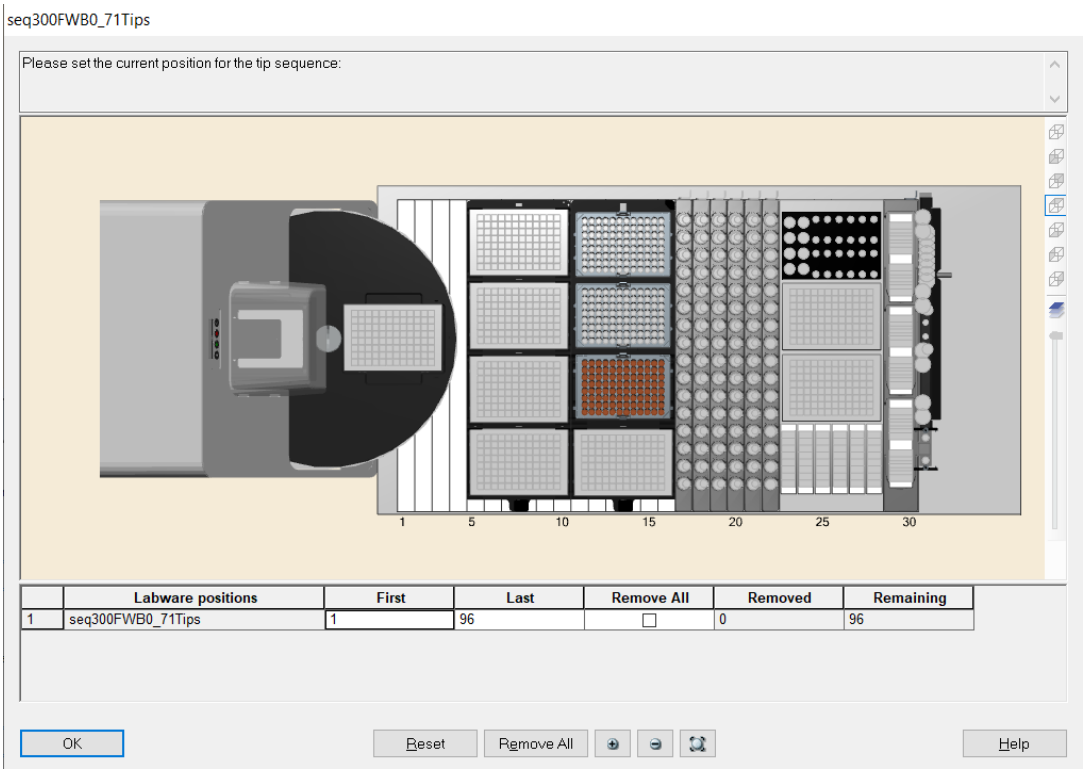
11. Use the cursor to set the first and last position of the 1000 µL conductive filter tips, then click “Ok”.



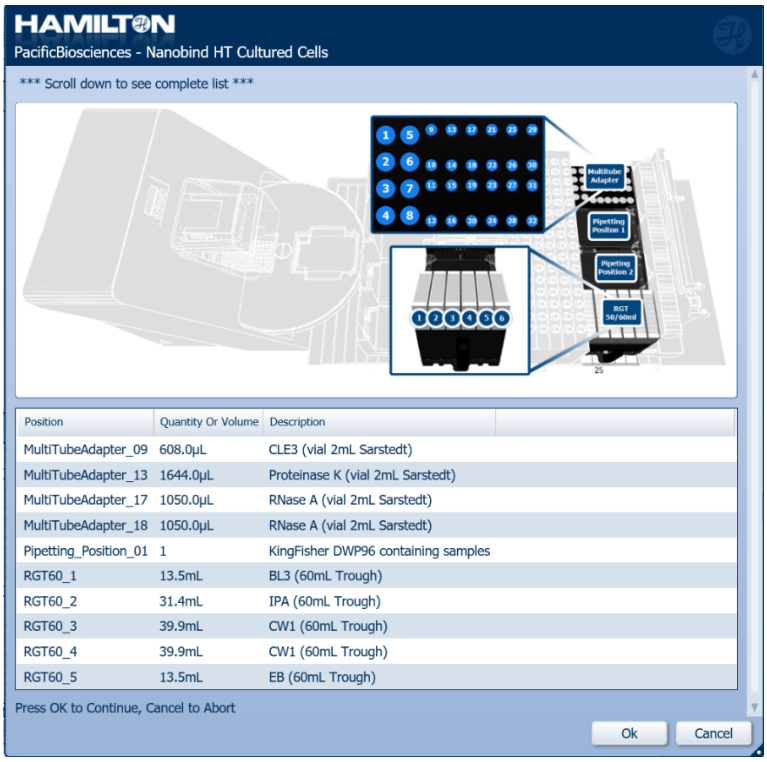
12. Use the cursor to set the first and last position of the 300 µL conductive filter tips, then click “Ok”.



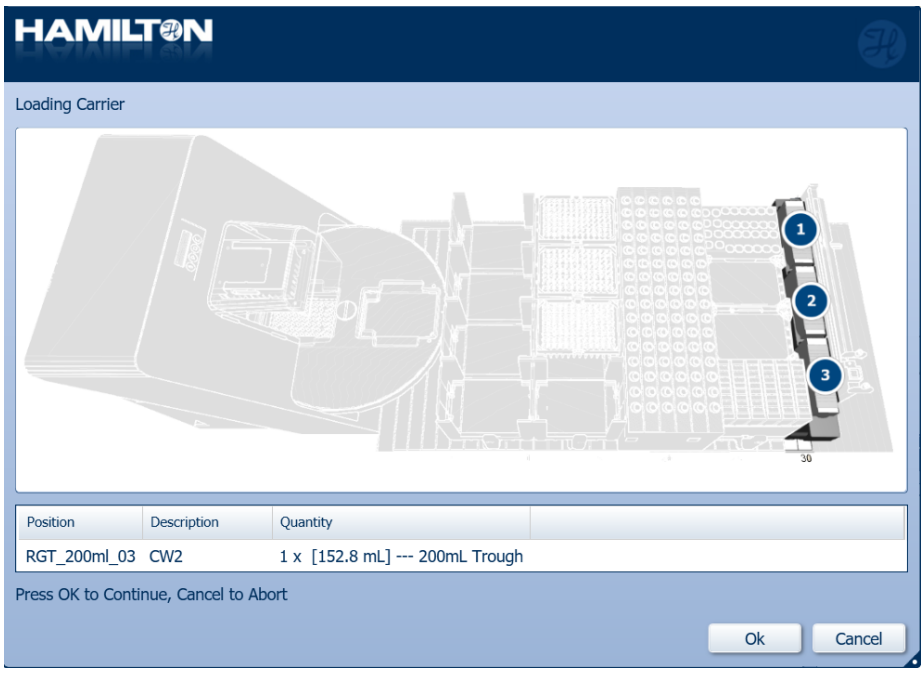
13. Use the cursor to set the first and last position of the 300 µL wide-bore conductive filter tips, then click “Ok”.



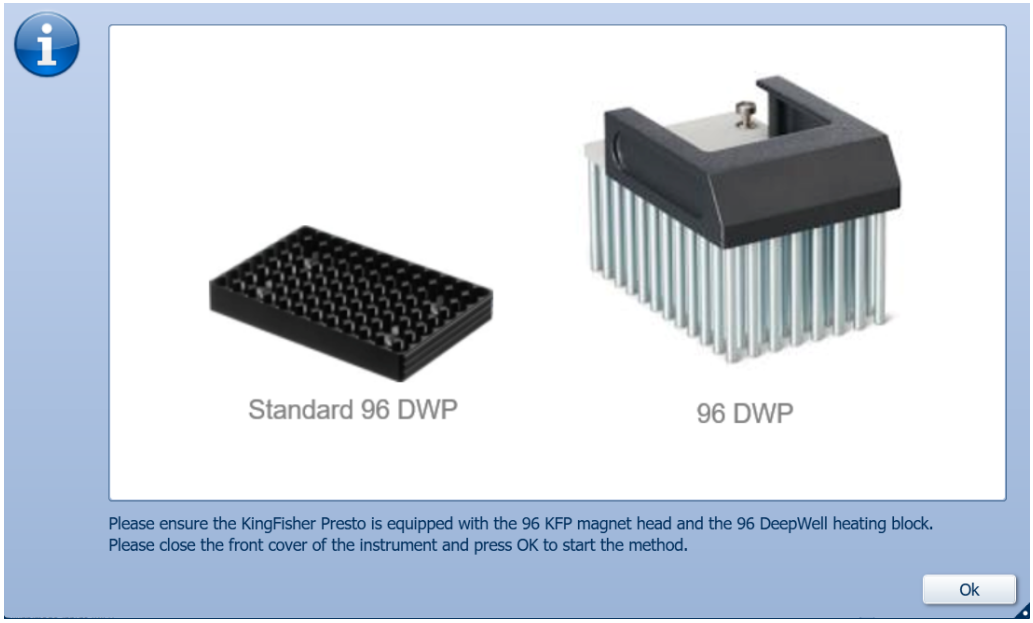
14. Fill the listed number of 2 mL screw cap micro tubes with the listed volumes of Proteinase K, Buffer CLE3, and RNase A, then load them on the listed positions in the MultiTube Adapter. Load the Sample Plate on the listed carrier position. Using a serological pipette, fill 60 mL reagent reservoirs 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”.



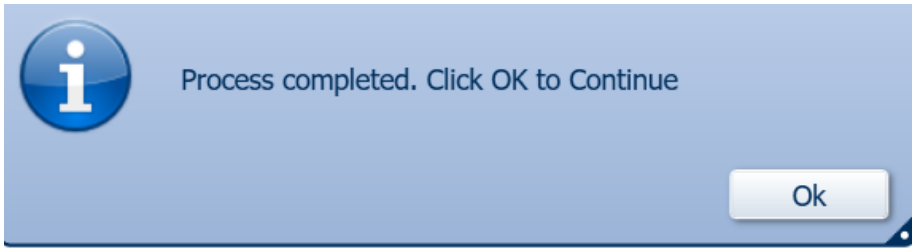
15. Fill one 200 mL reagent reservoir with the listed volume of Buffer CW2, then load it on the listed position of the reagent reservoir carrier. Then, click “Ok”.



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



17. At the end of the run (~160 minutes after start), click “Ok” to end the run.



18. Remove plates from the instrument.
19. 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.
20. 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.
21. Let eluate rest overnight at RT to allow DNA to solubilize.
    - Visible “jellies” should disperse after resting.
22. 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.

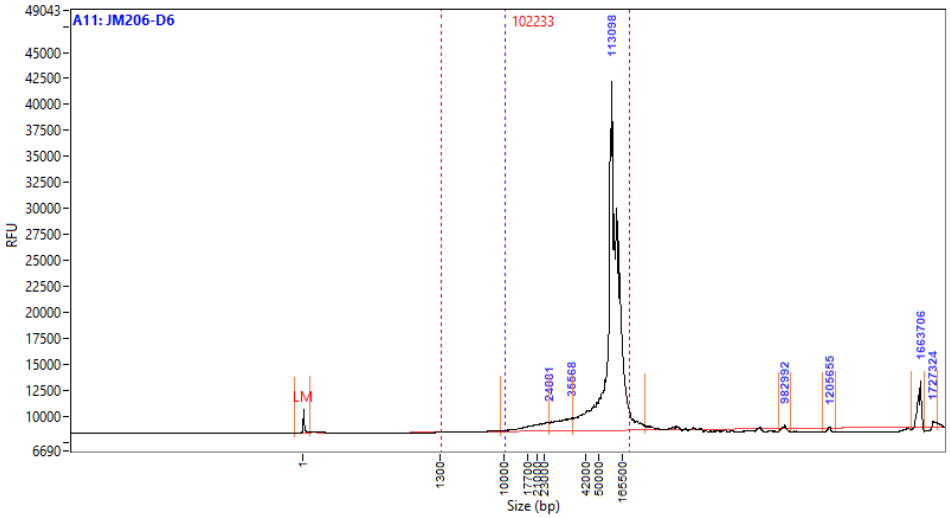
Storage of DNA

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 cultured Gram-negative and Gram-positive bacteria will yield ~1–10 µg.
- 260/280 ratios should consistently be 1.7–2.0.
- 260/230 ratio can vary from 1.2–1.8.
- 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 cultured Gram-negative and Gram-positive bacteria DNA measured on the Femto Pulse system (Agilent Technologies) is typically 100 kb+.

Sample	260/280	260/230	Nanodrop (ng/µL)	Qubit DNA yield (µg)
E. coli	1.79	1.26	53.62	4.74
S. enterica	1.90	1.60	117.39	5.54
S. sonnei	1.83	1.59	49.68	4.65
K. pneumoniae	1.82	1.47	125.74	5.15
P. aeruginosa	1.89	1.90	72.34	7.50
L. monocytogenes	1.78	1.22	151.54	9.78
E. faecalis	1.73	1.36	106.84	6.47
S. aureus	1.89	1.88	30.98	2.30



DNA size distribution of unsheared gDNA isolated from an *E. coli* 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.
   - High heterogeneity can be caused by insufficient mixing during the cell preparation steps. Ensure the cells have been fully resuspended in PBS and STET Buffer with lysozyme before adding to the Sample Plate.

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.
   - The cell input could be too low. For cultured Gram-negative and Gram-positive bacteria, we recommend $5x10^8$ cells. For example, $5x10^8$ *E. coli* cells should recover ~2-5 µg of DNA.
   - 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.
   - The lysis could be inefficient due to improper resuspension of the cell pellet prior to lysis. Make sure the cell pellet is completely resuspended during the cell preparation steps and no visible cell clumps remain. We recommend pipette-mixing as thoroughly as possible at step 3.
   - Some bacteria are difficult to lyse and could require additional reagents, other enzymes, or even mechanical lysis. Contact PacBio for questions about specific bacteria.

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, cultured Gram-negative and Gram-positive bacteria DNA results in UV purities of 260/230 >1.2 and 260/280 >1.7. 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.
   - The purity could be lower due to insufficient lysis resulting from too high of a cell input. We recommend inputs of $5x10^8$ cells. Inputs greater than this can overwhelm the lysis chemistry, resulting in lower recoveries and lower purity.

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, especially at high cell inputs (>25 µg yields). We recommend rerunning the protocol at a lower cell input if additional 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. **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