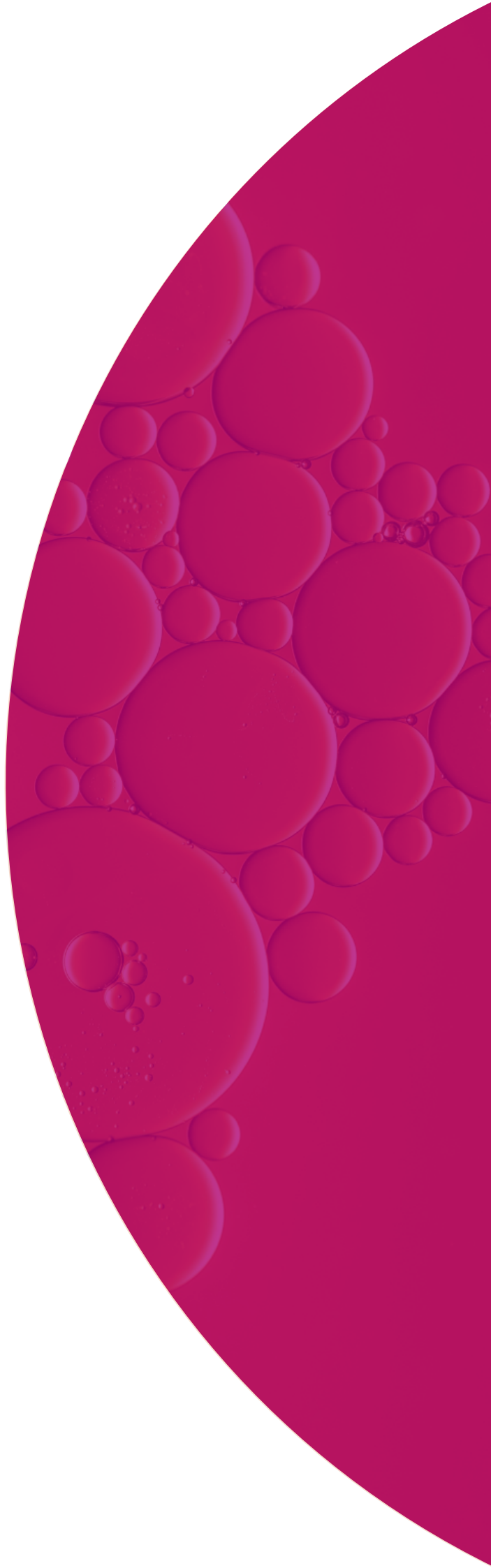


# Extracting HMW DNA using the Nanobind<sup>®</sup> HT CBB kit for cultured bacteria on the KingFisher Apex system

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 Thermo Fisher KingFisher Apex 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)
KingFisher Apex System	Thermo Fisher Scientific (5400930, includes Apex 96 deep well magnet head)
KingFisher Apex 96 deep-well magnet head	Thermo Fisher Scientific (24079930)
KingFisher Apex 96 deep-well heating block	Thermo Fisher Scientific (24075920)
KingFisher 96 deep-well plates, barcoded	Thermo Fisher Scientific (95040450B)
KingFisher 96 deep-well tip combs, barcoded	Thermo Fisher Scientific (97002534B)
Tris-HCl, 1 M, pH 8.0	Invitrogen (15568025)
Ethylenediaminetetraacetic Acid (EDTA), 0.5 M, pH 8.0	ThermoFisher 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

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## 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:  $5 \times 10^8$  Gram-negative or Gram-positive bacteria

- *E. coli* generally have  $5 \times 10^9$  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  $\mu\text{g}$  of DNA.
  - For *E. coli* and *L. monocytogenes*, this was 0.5 mL of 1 OD600 culture.
  - **Warning: cell inputs that yield >25  $\mu\text{g}$  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

- Ensure the proper protocol has been installed on the KingFisher instrument (see **Nanobind HT kit Guide & overview “Programs”**).
- 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  $\mu\text{L}$  lysostaphin to 50  $\mu\text{L}$  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 Thermo Fisher KingFisher Apex system. 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.

**This protocol is not intended for use with the Thermo Fisher KingFisher Apex 96 combi tip comb. Use the 96 deep-well tip comb with the 96 deep-well magnetic head. Failure to do so will result in subpar performance, including reduced recovery, reduced purity, and increased rate of hard failures (e.g. lost or dropped disks).**

1. Collect 7 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	Lysis/Binding	Sample + reagents from Step 2	
2	Nanobind Storage Plate	One 3 mm Nanobind Disk per well*	
3	Wash Plate 1	Buffer CW1	700 $\mu$ L
4	Wash Plate 2	Buffer CW2	700 $\mu$ L
5	Wash Plate 3	Buffer CW2	700 $\mu$ L
6	Elution Plate	Buffer EB	100 $\mu$ L
7	Tip Plate	KingFisher Apex 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  $5 \times 10^8$  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  $\mu$ g of DNA.
  - **Warning: cell inputs that yield >25  $\mu$ g may result in Nanobind disks being “dropped” in the Lysis/Binding solution and/or cause well-to-well contamination.**
3. Add 10  $\mu$ L 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  $\mu$ L 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  $\mu$ L lysostaphin to 50  $\mu$ L STET buffer + lysozyme per extraction.
5. Prepare sample in the Lysis/Binding Plate (Plate 1):
  - Add the samples prepared in Steps 2-4 to individual wells.
  - Add 10  $\mu$ L of Proteinase K to each well.

- Add 5  $\mu$ L of Buffer CLE3 to each well.
- Add 20  $\mu$ L of RNase A to each well.

Note: the sample and reagents MUST be added to the wells in the order described above.

Note: reagents MUST be added directly into the liquid rather than against the side of the well.

6. Ensure the instrument is set up with the 96 deep-well magnetic head and the 96 deep-well heating block.
7. Insert the 96 deep-well tip comb into a 96 deep-well plate (Plate 7, Tip Plate).
8. Select the **Bacteria\_Nanobind\_HT\_APEX** script on the KingFisher Apex instrument and press 'Start'.
9. Insert plates into the KingFisher Apex instrument as indicated on the display and press 'Next' after every plate to confirm position.
10. The protocol will start when the final plate is loaded and the 'Next' button is pressed.
11. When prompted by the instrument (~35 minutes after start), remove the Lysis/Binding plate (Plate 1) from the instrument and add 100  $\mu$ L of Buffer BL3 to each well. Re-insert the plate and press 'Next' to resume the protocol.  
**Note: add BL3 gently against the side of the well into the Lysis/Binding solution. Adding BL3 directly to the Lysis/Binding solution may affect extraction performance.**
12. When prompted by the instrument (~56 minutes after start), remove the Lysis/Binding plate (Plate 1) from the instrument and add 250  $\mu$ L of isopropanol to each well. Re-insert the plate and press 'Next' to resume the protocol.  
**Note: add isopropanol gently against the side of the well into the Lysis/Binding solution. Adding isopropanol directly to the Lysis/Binding solution may affect extraction purity.**
13. At the end of the run (~100 minutes after start), remove plates as indicated on the instrument display. The protocol will end when the final plate is removed and the 'Next' button is pressed.
14. Transfer eluates from the Elution Plate (Plate 6) to a new storage plate or storage tubes.
  - 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 (Plate 7) after elution. This does not affect extraction performance.
  - When transferring eluate, a small amount of liquid may remain on the Nanobind disk. Use a P200 pipette tip to transfer any liquid remaining on the Nanobind disk.
15. 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.
16. Let eluate rest overnight at RT to allow DNA to solubilize.
  - Visible "jellies" should disperse after resting.
17. 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

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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

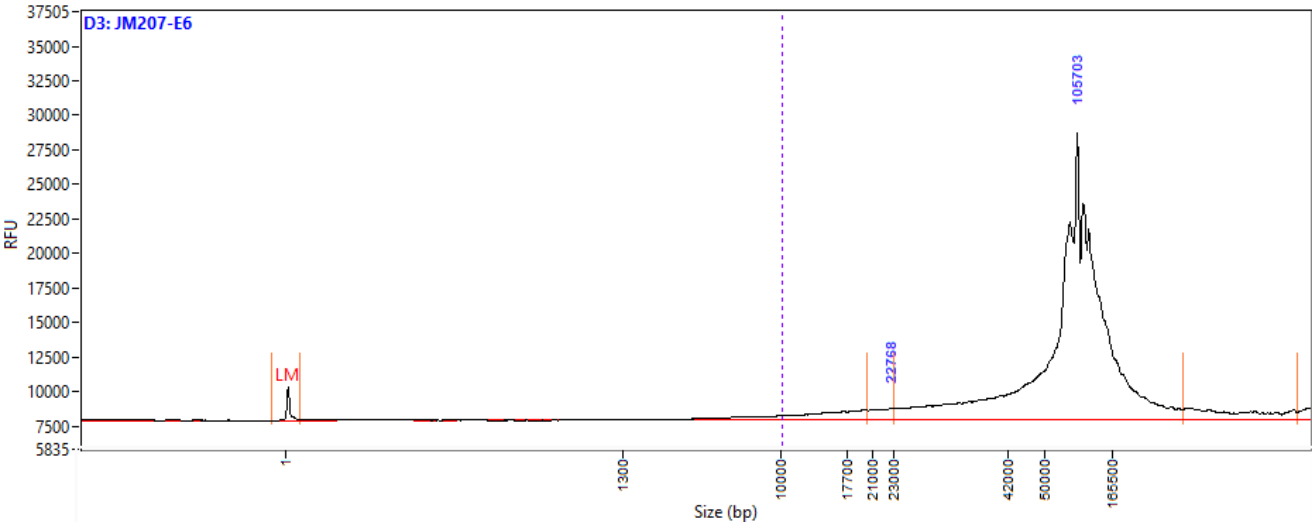
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DNA can be stored in Buffer EB at 4°C for several months. Long term storage at -20°C or -80°C can be used if necessary. Avoid freeze/thaw cycles since this can degrade high molecular weight DNA.

# 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)
<i>E. coli</i>	1.84	1.70	111.52	5.83
<i>S. enterica</i>	1.93	1.66	69.87	5.84
<i>S. sonnei</i>	1.84	1.76	117.98	5.45
<i>K. pneumoniae</i>	1.90	1.62	63.60	4.59
<i>P. aeruginosa</i>	1.87	1.79	125.30	6.36
<i>L. monocytogenes</i>	1.76	1.24	85.99	5.14
<i>E. faecalis</i>	1.70	1.33	54.60	3.72
<i>S. aureus</i>	1.87	1.75	57.14	5.57



DNA size distribution of unsheared gDNA isolated from an *E. coli* sample using the KingFisher Apex on the Femto Pulse system (Agilent Technologies).



# Troubleshooting FAQ

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## 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 Lysis/Binding 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  $5 \times 10^8$  cells. For example,  $5 \times 10^8$  *E. coli* cells should recover ~2–5  $\mu\text{g}$  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  $5 \times 10^8$  cells. Inputs greater than this can overwhelm the lysis chemistry, resulting in lower recoveries and lower purity.

- If purities are generally low across all samples, ensure the isopropanol was added as recommended in Step 12 (add isopropanol so it gently trickles down the side of the well into the Lysis/Binding solution). Adding isopropanol directly to the Lysis/Binding solution can result in decreased purities.

**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 Plate 7 (Tip Plate) at the end of the protocol. If the Nanobind corresponding to the sample in question has been returned to Plate 7, 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 Plate 7 (Tip Plate), this sample will likely not contain any DNA. Occasionally, the disk can become dislodged from the magnet during binding and remains in the Lysis/Binding plate (Plate 1). 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 on the tip comb in Plate 7 and not in the Lysis/Binding plate (Plate 1), 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. The instrument will give an error message if the correct magnet head for a given protocol is not installed.
- Check to ensure all consumables are in the correct positions. The instrument 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 KingFisher Apex uses barcoded 96 deep-well sample plates (Thermo Fisher 95040450B). The instrument will give an error message if it does not detect a barcode. Hitting "OK" will allow the script to continue (it will still work with the non-barcoded version of the plate). If an incorrect plate or tip comb barcode is detected the instrument will display an error message (for example, if a 24 deep-well plate was inserted instead of a 96 deep-well plate).
- For other on-instrument error messages, contact Thermo Fisher.

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

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