BEFORE STARTING

Prepare between 11 and 95 RNA samples plus 1 negative control using this protocol.

1. END PREPARATION REACTION

1.1. In a new PCR strip-tube/plate set up the following reaction for each sample:

Component	Volume
Cleaned-up PCR from previous step	5 µL
Ultra II End Prep Reaction Buffer	1.2 µL
Ultra II End Prep Enzyme Mix	0.5 µL
Nuclease-free water	3,3 µL
Total	10 µ L

Make a master mix of end-preparation reagents and nuclease-free water and aliquot into strip-tube/plate to improve reproducability.

1.2. Incubate at room temperature for 00:15:00
Incubate at 65 °C for 00:15:00
Incubate on ice for 00:01:00

2. BARCODE LIGATION

2.1. In a new PCR strip-tube/plate set up the following reaction for each sample:

Component	Volume
End-preparation reaction mixture	0.75 µL
NBXX barcode	1.25 µL
Blunt/TA Ligase Master Mix	5 µL
Nuclease-free water	3 µL
Total	10 µ L

Use one native barcode from the EXP-NBD104 (1-12), EXP-NBD114 (13-24) or EXP-NBD196 per sample. Use 12 or more barcodes per library or there will be insufficient total material to achieve good yields.

2.2. Incubate at room temperature for 00:30:00
Incubate at 65 °C for 00:15:00
Incubate on ice for 00:01:00

The 65°C incubation is to inactivate the DNA ligase to prevent barcode cross-

ligation when reactions are pooled in the next step

2.3. In a new 1.5 mL Eppendorf tube pool all one-pot barcoding reactions together.

If processing 12-24 samples pool all 10 µl from each native barcoding reaction.
If processing 48 samples pool 5 µl from each native barcoding reaction.
If processing 96 samples pool 2.5 µl from each native barcoding reaction **so as not to exceed a pool volume of 240 µl which would make the clean-up volume too large.**

2.4. Add 0.4x volume of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add 192 µl SPRI beads to 240 µl pooled one-pot barcoding reactions.

0.4x volume of SPRI is sufficient to bind 400 bp amplicons in the presence of ligation buffer, do not use 1x as this will result in an excessive large bead pellet.

2.5. Mix by vortexing and pulse centrifuge to collect all liquid at the bottom of the tube. Incubate for 00:05:00 at room temperature.

2.6. Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

2.7. Add 200 µl or more if you want to cover the pellet of room-temperature 80% volume ethanol to bathe the pellet. Carefully remove and discard ethanol, being careful not to touch the bead pellet.

Only perform 1x 80% ethanol wash.

2.8. Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much as residual ethanol as possible using a P10 pipette.

2.9. With the tube lid open incubate for 00:01:00 or until the pellet loses it´s shine (if the pellet dries completely it will crack and become difficult to resuspend).

2.10. Resuspend pellet in 31 µl nucleases free water, mix gently by either flicking or pipetting and incubate for 00:02:00.

2.11. Place on magnet and transfer sample to a clean1.5 mL Eppendorf tube ensuring no beads are transferred into this tube.

Quantify 1 µl of the barcoded amplicons using the Qubit dsDNA assay. Concentration will vary depending on number and Ct of samples and but you need about 30 ng total at this stage to acheive maximum run yield.

3. ADAPTER LIGATION

3.1. In a new 1.5µl Eppendorf tube set up the following AMII adapter ligation reaction.

Component	Volume
Barcoded amplicon pool	30 µL
Adapter Mix (AMII)	5 µL
Blunt TA	35 µL
Total	70 µ L

3.2. Incubate at room temperature for 00:20:00

3.3. Add 70 µl (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. Pulse centrifuge to collect all liquid at the bottom of the tube.

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown color.

There will be some variation in clean-up efficiencies but expect to carry around 50% through this clean-up

3.4. Incubate for 00:05:00 at room temperature.

3.5. Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

3.6. Add 250 µl SFB and resuspend beads completely by pipette mixing. Pulse centrifuge to collect all liquid at the bottom of the tube. Remove supernatant and discard.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

3.7. Repeat steps 4.6 to perform a second SFB wash.

3.8. Pulse centrifuge and remove any residual SFB. Add 16 µl EB (ONT) and resuspend beads by pipette mixing.

You do not need to allow to air dry with SFB washes.

3.9. Incubate at room temperature for 00:02:00 .

3.10. Place on magnetic rack until clear. Transfer final library to a new 1.5mL Eppendorf tube.

3.11. Quantify 1 µl of the final library using the Qubit dsDNA assay. Concentration will vary depending on number and Ct of samples but 15 ng final library is usually required to achieve maximum run yield.

The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook

4. MinION SEQUENCING

4.1. Prime the flowcell and load 15 ng sequencing library onto the flowcell

4.2. From experience we know 15 ng is optimum loading input for short amplicons. Speed drop during the run indicates excessive library was loaded. Low run yield <20M reads indicates insufficient library.

4.3. Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)

Loading beads (LB)

Flush buffer (FB)

Flush tether (FLT)

4.4. Add 30 µl FLT to the FB tube and mix well by vortexing.

4.5. If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

4.6. Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

4.7. Take a P1000 pipette and tip and set the volume to 800 µl. Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

4.8. Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

4.9. Load 800 µl of FB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

4.10. Wait for 00:05:00.

4.11. Gently lift the SpotON cover to open the SpotON port.

4.12. Load another 200 µl of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

4.13. In a new tube prepare the library dilution for sequencing:

Component	Volume
SQB	37.5 µL
LB	25.5 µL
Library	12 µL
Total	75 µ L

Mix LB immediately before use as they settle quickly.

Make up with EB if less than 12 µL library is required.

4.14. Mix the prepared library gently by pipetting up and down just prior to loading.

4.15. Add the 75 µl library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

4.16. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.

Start the sequencing run using MinKNOW.

4.17. If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

4.18. Choose flow cell 'FLO-MIN106' from the drop-down menu.

4.19. Then select the flowcell so a tick appears.

4.20. Click the 'New Experiment' button in the bottom left of the screen.

4.21. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit Selection: Select LSK109 and for native barcoding choose NBD196.

Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected).

Click 'Start run'.

4.22. Monitor the progress of the run using the MinKNOW interface.

Step-by-step MinION sequencing - 1D Native barcoding genomic DNA (with EXP-NBD104, EXP-NBD114, and SQK-LSK109)

REVISÃO: 1
DATA: 24/Setembro/2020

The **sequencing** protocol must be performed in a post-PCR area. The area and glassware and plastic ware must be cleaned previously with 1% sodium hypochlorite solution, surface decontaminant DNase (DNA Away) and 70% EtOH.

IMPORTANT

You MUST have AT LEAST ONE negative control on EVERY library. Ideally, the negative control should follow the same steps of other samples (extraction/cDNA/PCR steps), but if these are unexpectedly unavailable then you should include a water control instead at this stage. This is not ideal, but you MUST have a negative control.

BEFORE STARTING:

- Take AMPure beads out the fridge and mix by vortexing gently. Use at room temperature.
- Make 1ml stocks of AMPure beads and nuclease-free water to avoid contamination.
- Prepare 70-80% EtOH in a falcon tube.
- Label the 1.5mL LoBind tubes with the ID of the previously quantified samples.
- Mix by inversion and spin down all reagent tubes before using.

NOTE: DON'T LEAVE THE REAGENTS OUT OF THE FREEZER WHEN NOT IN USE

After the quantification of all purified samples, we need to normalize the amount of each sample used as input to the barcoding library preparation.

1. PCR products purification

Perform SPRI clean-up by doing the **Step-by- step Clean-up**:

a) Add 1x **AMPure beads** previously homogenized at Room temperature to each tube;

b) Mix by inversion for **5** minutes to promote the binding of the library to the beads;

c) Spin down quickly and place on a magnetic rack;

d) Once the beads have pelleted and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet avoiding to touch the pellet. Discard the supernatant;

e) With the tube still on the magnetic rack, wash the pellet with **200 µl** of **80% ethanol** (or you may need more ethanol to get good wash if lots of beads). Do not disturb the pellet; Pipette carefully;

f) Discard the ethanol and wash again with **200** µl of **80% ethanol** (or you may need more ethanol to get good wash if lots of beads). After discarding the second time, close the tubes, spin down and place again on magnetic rack;

g) Remove carefully the residual ethanol with a 10 or 20 pipette, then leave open to air dry until the pellet loses its shine (If the pellet is large, you can speed up drying by briefly incubating at **65 °C**; do not allow the pellet to overdry and crack, or recovery will be reduced.)

h) When the pellet is already dry (no shining against the light), elute the pellet by adding **21 µl** of nuclease-free water (NFW) and resuspend the beads by flicking or with the pipette, make sure all beads have been eluted from the wall tube;

i) Incubate for **10** minutes at **37°C**;

j) Spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the eluate to a new tube, previously labelled. (**Note:** be careful to not transfer the beads to the new tube).

2. DETERMINE THE NUMBER OF SAMPLES PER FLOW CELL

3. NORMALIZATION OF DNA CONCENTRATIONS

- Divide the total input quantity (240 or 500ng of DNA) by the number of barcodes being used to calculate the quantity per barcode not taking into account the negative controls. The negative controls does not enter in the dilution accounts, but they will be counted as a barcode later.

- If using an amplification protocol based on pools scheme, add the appropriate volume of each sample pool A and pool B to individual labelled 1.5ml Eppendorf tubes (i.e., mixing pool A and pool B at this stage for each sample) and then adjust the volume in each Eppendorf to 20 µl with nuclease-free water.

Input DNA

$$\frac{\frac{Total\ input}{n^o\ of\ barcodes}}{2} = X\ ng\ per\ pool\ per\ sample$$

For each sample Pool A:

$$\frac{X\ ng}{Qubit\ result\left(ng/\mu L\right)} = Y\ \mu L\ of\ sample\ pool\ A$$

For each sample Pool B:

$$\frac{X\ ng}{Qubit\ result\left(ng/\mu L\right)} = Z\ \mu L\ of\ sample\ pool\ B$$

For each sample, add the following volume of NFW to have a final volume of *20 µl* (at this point you should have a single tube containing pool A and B):

$$20\ \mu L - Y\ \mu L - Z\ \mu L = Vol.\ of\ NFW\ needed$$

4.END-REPAIR AND dA-TAILING:

Component	Amount (µl)
Normalized amplicons (**from Step 3)**	20
Ultra II End Prep Reaction Buffer	2.8
Ultra II End Prep Enzyme Mix	1.2
Total	24µl

- Add the above volumes of Ultra II End Prep Reaction Buffer and Ultra II End Prep Enzyme Mix to each normalized amplicon tube. Mix by inversion, spin down and incubate for **10 min** at Room Temperature, followed by **5 min** at **65** ℃.

- Perform SPRI clean-up by doing the **Step-by- step Clean-up** (**Step 1**)

a) Add **1x AMPure beads** previously homogenized at Room temperature to each tube;

b) Mix by inversion for **5 minutes** to promote the binding of the library to the beads;

c) Spin down quickly and place on a magnetic rack;

d) Once the beads have pelleted and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet avoiding to touch the pellet. Discard the supernatant;

e) With the tube still on the magnetic rack, wash the pellet with **200 µl of 80% ethanol** (or you may need more ethanol to get good wash if lots of beads). Do not disturb the pellet; Pipette carefully;

f) Discard the ethanol and wash again with **200 µl of 80% ethanol** (or you may need more ethanol to get good wash if lots of beads). After discarding the second time, close the tubes, spin down and place again on magnetic rack;

g) Remove carefully the residual ethanol with a 10 or 20 pipette, then leave open to air dry until the pellet loses its shine (If the pellet is large, you can speed up drying by briefly incubating at 65 ℃; ***do not allow the pellet to overdry and crack***, or recovery will be reduced.)

h) When the pellet is already dry (no shining against the light), elute the pellet by adding **11 µl** of **NFW** and resuspend the beads by flicking or with the pipette, make sure all beads have been eluted from the wall tube;

i) Incubate for **10 minutes** at **37°C**;

j) Spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the **eluate** to a new tube (Note: be careful to not transfer the beads to the new tube).

5. NATIVE BARCODE LIGATION

Before starting this step, organize and record the order of your samples with the order of the barcodes.

Example: Sample 1 (A and B mixed) – Barcode 1
Sample 2 (A and B mixed) – Barcode 2 ……..

IMPORTANT: the negative control should be placed randomly within the library (e.g., do not always use BC12 or BC24 for the negative control). When we are running with the nuclease flush, it would be much harder to detect if there was any cross-library contamination if the negative control was always on the same barcode. Please make sure that the negative control is placed in a different barcode for each library.

NOTE: be careful in this step to do not change the order of samples and barcodes. During the analysis, these notes will be necessary to know which samples correspond to each barcode.

Component	Amount (µl)
dA-tailed amplicons (from Step 4)	10
Native barcode NB01-NB24	2.5
Blunt TA	12.5
Total volume	25

- Mix by inversion, spin down and incubate at room temperature (20 °C) for **15** min, followed by **65** °C for **20-30** min to denature the ligase.

- *Combine all* the barcode ligation reactions (all the tubes) into a **single** 1.5-ml Eppendorf tube (you can use the same tip). Alternatively, put half in one tube and half in another.

- Perform SPRI cleanup by doing the **Step-by- step Clean-up**.

a) Add **1x AMPure beads** previously homogenized (in this case: **25 µl * n°** barcodes included in the tube);

b) Mix by inversion for **5 minutes** to promote the binding of the library to the beads;

c) spin down and place on a magnetic rack; once the beads have pelleted and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet to avoid to touch the pellet. Discard the supernatant;

d) with the tube still on the magnetic rack, wash the pellet with **400 µl of 80% ethanol** (or you may need more ethanol to get good wash if lots of beads). Do not disturb the pellet; Pipette carefully;

e) discard the ethanol and wash again with **400 µl of 80% ethanol** (or you may need more ethanol to get good wash if lots of beads). After discarding the second time, close the tubes, spin down and place again on magnetic rack;

f) remove the residual ethanol then leave open to air dry until the pellet loses its shine (If the pellet is large, you can speed up drying by briefly incubating at 65 °C; ***do not allow the pellet to overdry and crack***, or recovery will be reduced.)

g) When the pellet is already dry (no shining against the light), elute the pellet by adding **46 µl** of **NFW** and resuspend the beads by flicking or with the pipette, make sure all beads have been eluted from the wall tube;

h) Incubate for **10** minutes at **37°C**;

i) Spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the **eluate** to a **new** tube. (Note: be careful to not transfer the beads to the new tube).

Quantify **1 µl** combined barcoded amplicon pools on the Qubit to confirm that not much DNA was lost during the purifications.

6. Adapter ligation and clean-up.

Set up the following ligation reaction, mixing between each addition.

Component	Amount (µl)
Pooled barcoded sample (from Step 5)	45
Adapter Mix (***AMII***)	5
Blunt TA	50
Total volume	100

a) Mix by inversion, spin down and incubate at Room Temperature for **30 minutes (<u>In the meantime, perform the flow cell Quality Control</u>)**.
b) Add **100** µl of AMPure beads previously homogenized;
c) Mix by inversion for **5 minutes** to promote the binding of the library to the beads;
d) Spin down and place on the magnetic rack;
e) Once the beads have pelleted and the liquid is completely clear, remove the supernatant with the 100 or 200 pipette by the opposite side of the pellet to avoid to touch the pellet. Discard the supernatant;
f) With the tube still on the magnetic rack, wash the pellet with **<u>250 µl of *SFB*</u>** ***(short fragment buffer)***. Close tube lid, resuspend beads by flicking.
g) Spin down, beads on magnet and remove supernatant - discard the supernatant and wash again with **250** µl of ***SFB***. After discard the second time, close the tubes, spin down and place again on magnetic rack;
h) Remove the residual **SFB** and then elute the pellet by adding **13 µl of <u>*EB*</u>** (Elution Buffer) and resuspend the beads by flicking or pipetting, make sure all beads have been eluted from the wall tube;
i) Incubate for **10** minutes at **37°C**;
j) Spin down, place on the magnetic rack, let the pellet beads on magnet, transfer the **eluate** to a new tube. (Note: be careful to not transfer the beads to the new tube).

Quantify **1 µl** of your eluted library using the Qubit. With the new R9.4.1 you only need **10 - 30 ng** to sequencing. So, after quantifying, dilute the library to **30** ng in **12 µL,** using ***<u>EB</u>*** to dilute.

7. PRIMING AND LOADING THE SpotON FLOW CELL

- Thaw the following reagents at room temperature before placing the tubes on ice as soon as thawing is complete:
Sequencing Buffer (***SQB***)
Loading Beads (***LB***)
Flush Tether (***FLT***)
Flush Buffer (***FLB***)

- Mix the Sequencing Buffer (**SQB**) and Flush Buffer (**FLB**) tubes by vortexing, spin down and return to ice.
- Spin down the Flush Tether (**FLT**) tube, mix by pipetting, and return to ice.

Prepare the Priming mix:
- Add **30 µL** of thawed and mixed Flush Tether (**FLT**) **directly** to the tube of thawed and mixed Flush Buffer (**FLB**)and mix by pipetting up and down.

- Take out a flow cell from the fridge.
- Open the sample port. Draw back with the P1000 a few µL(~10 µL) of buffer to make sure there is continuous buffer flow from the sample port across the sensor array and that there are no bubbles in the flow cell.

- Load **800 µL** of the **priming mix** slowly through the sample loading port using a P1000 pipette. <u>It is extremely important not to introduce or push any air bubbles into the flow cell.</u>
- Wait **5 minutes**.

- Gently lift the **SpotON port** cover to make the sample port accessible.
- Load **200 µL** of the priming mix as before.

Prepare the library for loading:

Component	Amount (µl)
Sequencing Buffer (**SQB**)	37.5
Loading Beads (**LB**), mixed immediately before use	25.5
DNA library (30 ng)	12
Total volume	75

a) Mix gently by pipetting and spin down.
b) Add the **75 µL** diluted library to the flow cell by allowing droplets to fall onto the **SpotON port** using a **P200** pipette without the pipette touching the port. Droplets will be drawn into the flow cell by capillary action. If the library is not drawn into the flow cell, close the SpotON port and perform a further 200 µL prime via the sample inlet port and try loading the library again.
c) Gently replace the **SpotON port** cover, making sure the bung enters the SpotON.

8. START SEQUENCING RUN

By default, you will need an Internet connection before the sequencing script can be started, although off-line versions of MinKNOW can be requested from the manufacturer if an Internet connection is not available.

Note: the starting voltage can vary depending on how long you have previously run that flow cell for. Approximate run voltages are: for the first 10 hours -180 v, after 10 hours -190 v, after 200v.

9. FLOW CELL DIGESTION

After sequencing, set up the following **digestion buffer**, mixing between each addition (*this an adapted wash protocol using reagents from old sequencing kits. For new sequencing kits please use the proper wash kit provided by ONT*).

Component	Amount (µl)
Running buffer (RBF)	100
Nuclease free water	100
CaCl2 (0.1M – dilute 1M stock 1/10)	4
DNase I	4
Total volume	208

a) Mix gently by pipetting and spin down.
b) Prepare a solution of diluted **RBF** (ratio 576 RBF + 672 NFW but you can make less).
c) Remove the waste from the flow cell.
d) Remove the air by pipetting from the sample inlet port.
e) Add **200 µL** of diluted ***RBF*** to the sample inlet port;
f) Add **150 µL** of the **digestion buffer** to the sample inlet port as well. <u>It is extremely important not to introduce or push any air bubbles into the flow cell.</u>
g) Let the flow cell digesting **overnight.**
h) Following digestion, check whether the flow cell is still generating reads by simulating a sequencing run. Check the reads and the active pores (usually you can see a high active pore number, but check if these are just the adapters).
i) After this step, add the Storage buffer following the official wash protocol from ONT. Then, you can keep the flow cell in the fridge or Room Temperature until the next sequencing run.