Protocol for Metagenomics Nanopore sequencing Last updated 22/09/24

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Primers

Sol-Primer A: 5'-GTTTCCCACTGGAGGATA-N9-3'
Sol-Primer B: 5'-GTTTCCCACTGGAGGATA-3'

Pre-treatment

Sample pre-treatment is dependent on both sample type, quality, clinical question and viral target. It is generally a good idea to perform centrifugation at 16,000 xG for 10 minutes and carefully (without disturbing the pellet) remove some supernatant. Below is an example for fecal samples:

Fecal sample pre-treatment:

1. Add 1250µl of PBS into a tube
2. Add one/two small scoops of fecal matter into a fresh tube and homogenize carefully
3. Vortex well to ensure a homogenous mix
4. Centrifuge for 10 minutes @ 16,000 x g
5. Carefully transfer 200µl of supernatant into a new tube

Nucleic acid extraction: Example for High Pure Total Viral Nucleic Acid extraction kit (Roche)

Sample extraction (Roche :

1. Add 50 µl of Proteinase K to 200 µl of sample and mix
2. Add 200 µl Binding Buffer and **mix immediately**
3. Incubate for 5 mins at 65'C (300 rpm if possible), then quick spin to remove condensation from the lid
4. Check the buffers have been prepared
5. Add 100 µl of binding buffer and mix well
6. Add the sample into the high pure filter tube inside the collection tube
7. Centrifuge at 0.3xG for 2 minutes (slow spin to increase yield)
8. Centrifuge at 8000xG for 15 seconds, discard the flow through and place in new collection tube
    a. **Optional:** To increase RNA viral sensitivity (**depends on sample type, aims and viral target e.g. DNA or RNA virus in mind**)

DNase Master Mix	1x	x
Turbo Buffer	88 µL	
Turbo enzyme	12µL	
Total	100 µL	-

    b. Add to the filter tube and incubate for 15 mins @RT
9. Add 500 µl of inhibitor removal buffer
10. Centrifuge at 8000xG for 1 minute, discard the flow through and place in new collection tube
11. Add 500 µl of wash buffer
12. Centrifuge at 8000xG for 1 minute, discard the flow through and place in new collection tube
13. Add 450 µl of wash buffer

14. Centrifuge at 8000xG for 1 minute, discard the flow through and place in new collection tube
15. Centrifuge at 13,000xG for 10 secs, discard the flow through and place in new collection tube
16. Open all lids and let ethanol evaporate for a moment
17. Add 50 µl of elution buffer and incubate for 5 min
18. Centrifuge at 8000xG for 1 minute, retain 50 µl elute. Store at -80°C.

Reverse transcription and random amplification

RT-PCR: (Benchtop fume hood)

Reagent	One reaction	x
Sol-Primer A (50µM)	0.5	
dNTP (12.5mM)	0.5	
Total	**1**	-

Add 1ul of master mix to 7.5ul of RNA or total nucleic acid

Reagent	One reaction
Nucleic acids	7.5

Program: (**Lid temperature ON**, preheated 70°C - 105°C)

- 1 min. at 70°C
- 4 min. at 65°C
- Snap cool on ice for 5 min and keep cool on a cold block if available or cool at room temperature

Reagent	One reaction	x
First strand buffer (5x) SSIV	2	
DTT (100 mM)	0.5	
Superscript IV (200 U/µl)	0.5	
Total	**3 µl**	-

Add 3 µl of first strand master mix to 8.5ul of randomly primed nucleic acid and mix (on cold block if available)

Program: (**Lid temperature ON @ 105`C**)

- 5 min. at 25°C
- 20 min. at 50°C
- 56°C 2 min
- 65°C 2 min
- 70°C 2 min
- 4°C hold in the PCR machine

Reagent	One reaction	x
Sequenase Buffer (5x)	0.6	
Sequenase Enzyme	0.4	
Total	**1µl**	-

Carefully add 1 µl of second strand master mix to 11.5ul of first strand product (on cold block).
Mix well and quick spin

Program: (**Lid temperature off!**)

- 4°C hold
- 37°C for 10 min
- 4°C for 1 min
- 37°C for 10 min
- 4°C hold

Second strand: (Benchtop fume hood)

Reagent	One reaction	x
LongAmp Taq (2x)	25	
Sol B primer (100 µM)	1	
Water	18	
Total	**44 µl**	-

Add 44ul of PCR master mix with 6ul of cDNA on a cold block

Reagent	One reaction
cDNA	6
Total	**50 µl**

Program: SISPA_SOLB (approx. 3 hours)

94°C for 1 min

94°C for 15 sec
55°C for 40 sec
65°C for 4 min
} x30

65°C for 10 min

8°C hold

PCR purification with Ampure XP beads: (approx. 30 mins) (Benchtop fume hood)

1. Allow the AMPure XP Beads to warm up to room temperature for 30 minutes and vortex the beads for 10 seconds.
2. Add 40µl beads (**0.8:1 ratio**) to the 50µl of sample and re-suspend with pipette.
3. Incubate the samples at RT for 5 min.
4. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
5. Carefully remove and discard the supernatant
6. Keep the sample on the magnet and add 200 µl of 80% ethanol. Incubate at RT for ≥30 sec
7. Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads. Repeat step 6-7 once.
8. Allow to dry at 30sec RT or until the beads are dry.
9. Remove the samples from the magnet
10. Re-suspend using 20µl of PCR-grade water.
11. Incubate at RT for 5 minutes.
12. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.

13. Carefully remove 19µl of the supernatant and transfer it to a new PCR plate with the date labeled cleaned SISPA cDNA
14. Take out Ultra II End-prep reaction buffer to defrost at RT, then place in the fridge

*Safe stopping point: can store cDNA in fridge overnight, otherwise -20 freezer

Qubit:

1. Prepare the Qubit mix 1/200.
2. Dilute the standards (1 = low and 2 = high) and samples
3. Standards: 190ul Qubitmix + 10ul standard
4. Samples: 199µl Qubitmix and 1µl sample
5. Measure the concentration on the Qubit. Click HOME choose: hsDNA (high sensitivity) program.

*The negative control should be less than 3ng/ul. If the negative is more than 3ng/ul, perform a 1/10 dilution

*The sample should be more than 5ng/ul. If the sample is under 2ng/ul, it is unlikely it will result in enough reads, but if it's an important sample take along. You can always upscale the master mix if you need to add more sample volume.

Native Barcoding Kit 24 V14 (SQK-NBD114.94): DNA repair and end-prep (Benchtop fume hood)

Reagent	One reaction	x
Ultra II End-prep reaction buffer	1.75	
Ultra II End-prep enzyme mix	0.75	
Total	**2.5 µl**	-

Add 2.5ul buffer enzyme mix/ sample to 12.5ul of sample

Reagent	One reaction
DNA/cDNA (100ng)	12.5
Total	**15 µl**

if you have less than 4 samples, use 200ng of cDNA input and double master mix volumes

PCR machine: 7.5 minutes 20°C, 7.5 minutes 65°C. Do not vortex the enzyme mix.

1. Thaw native barcodes at room temperature, spin down for 5 min, and put in fridge
2. Add 12ul (ratio 0.8:1) of re-suspended AMPure XP beads to the end-prep cDNA.
3. Incubate at RT for 5 min. to allow the sample to bind to the beads. Spin down the sample.
4. Place the samples in a magnetic particle collector to capture the beads.
5. Carefully remove and discard the supernatant
6. Keep the sample on the magnet and add 200 ul of 80% ethanol.
7. Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads. Repeat step 6-7 once.
8. Seal the plate. Spin down the sample and pipette off any residual supernatant.
9. Allow to dry at RT for ~30 seconds or until the beads are dry.
10. Remove the samples from the magnet
11. Re-suspend the DNA using 11µl PCR-grade water.
12. Seal the plate. Incubate at RT for 2 minutes. Spin down.

13. Place the samples in a magnetic particle collector to capture the beads. Carefully remove 10µl of the supernatant and transfer it to a new PCR plate (called "SISPA End-prep cleaned + date).

Native Barcoding Kit V14 (SQK-NBD114.96): Native barcode ligation (Benchtop fume hood)

Reagent	One reaction
End-prepped sample	10
Native Barcode	2.5
Blunt/TA Ligase Master Mix	12.5
Total	**25 µl**

Mix by pipetting and incubate for 20 minutes at room temperature.

1. Add 12.5ul (ratio 1:0.5) of re-suspended AMPure XP beads, re-suspend by pipetting
2. Incubate the samples at RT for 5 min. to allow the sample to bind to the beads. Spin down the sample. Will take longer to bind to the beads
3. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
4. Carefully remove and discard the supernatant
5. Keep the sample on the magnet and add 200 ul of 80% ethanol.
6. Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads. Repeat step 5-6 once.
7. Seal the plate. Spin down the sample and pipette off any residual supernatant.
8. Allow to dry at RT for ~30 seconds or until the beads are dry.
9. Remove the samples from the magnet.
10. Re-suspend the DNA using 11µl PCR-grade water.
11. Seal the plate. Incubate at RT for 10 minutes. Spin down
12. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear.
13. Carefully remove 10ul of the supernatant and transfer it to a new PCR plate called "SISPA BC cleaned + date.
14. Defrost/spin down and place in the fridge: NEBNext Quick Ligation Reaction Buffer (5x)

Qubit and Sample pooling:

1. Prepare the Qubit mix 1/200.
2. Dilute the standards (1 = low and 2 = high) and samples
3. Standards: 190µl Qubitmix + 10ul standard
4. Samples: 199µl Qubitmix and 1µl sample
5. Measure the concentration on the Qubit. Click HOME choose: hsDNA (high sensitivity) program.
   - For pooling and normalization, samples can be divided equally, or priority can be given to more important samples

Native Barcoding Kit V14 (SQK-NBD114.96): Adaptor ligation (Benchtop fume hood)

Reagent	One reaction
100 ng pooled barcoded sample	30
Native Adaptor (NA)	5
NEBNext Quick Ligation Reaction Buffer (5x)	10
Quick T4 DNA Ligase	5
Total	**50**

Mix and incubate for 20 mins at room temperature. Do not vortex the T4 ligase.

1. Take out ONT reagents
    a. SFB, elution buffer, LIB, FCT, FCF, SB, BSA – thaw at RT, **flick to mix and** quick spin. LIB, FCT, FCF, SB, BSA are then stored in the fridge until needed. SFB, elution buffer @ RT
    b. Remove a flow cell from the fridge
2. Add 25ul (ratio 1:0.5) of resuspended AMPure XP beads to the pooled barcoded sample, resuspend by pipetting
3. Incubate the samples at RT for 5 min to allow the sample to bind to the beads.
4. Spin down the sample.
5. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear (should take <1 min).
6. Carefully remove and discard the supernatant
7. Take the sample off the magnet and wash/re-suspend with 125ul short Fragment Buffer (SFB).
8. Incubate for 1 min then place back on magnet
9. Carefully remove and discard the supernatant. Try to remove everything without disturbing the beads. Repeat step 7-9 once.
10. Spin down the sample and pipette off any residual supernatant. – Do not let the beads dry out.
11. Remove the samples from the magnet
12. Re-suspend the DNA using 14 ul **elution buffer (EB).**
13. Incubate at room temperature for 10 minutes.
14. Place the samples in a magnetic particle collector to capture the beads. Incubate until the liquid is clear – at least 1 minute.
15. Carefully remove 13 µl of the supernatant and transfer it to a new DNA LoBind tube.
16. Use 1ul for Qubit (should have at least 12ul left)

Qubit:

1. Prepare the Qubit mix 1/200.
2. Dilute the standards (1 = low and 2 = high) and samples
3. Standards: 190ul Qubitmix + 10ul standard
4. Samples: 199µl Qubitmix and 1µl sample
5. Measure the concentration on the Qubit. Click HOME choose: hsDNA (high sensitivity) program.

Pooling:

1. Final concentration of
    a. 15-30ng in a total 12ul (make up with **elution buffer**) for R10 flowcell
    b. Can use to help with calculation: https://nebiocalculator.neb.com/#!/dsdnaamt
        i. mass-moles

ONT sequencing:

Prepare the following:

Reagent	One reaction
Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
Flow Cell Tether (FCT)	30 µl
Final total volume in Flow Cell Flush (FCF) tube	1,205 µl
Total	**1240 ul**

Prepare the following mix (vortex and spin down before use)

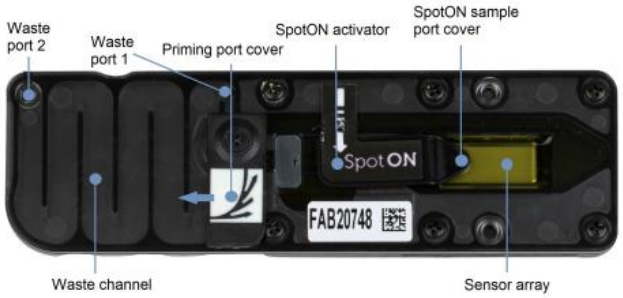
The library was prepared for loading:

Reagent	One reaction (µl)
SB (sequencing buffer)	37.5
LIB (loading beads mix before adding)	25.5
DNA library	12
Total	**75**

1. Place the flow cell in the MinIon
2. Flip back the flow cell lid and slide the priming port cover clockwise so that the priming port is visible
3. Draw back a small volume to remove any bubbles
    a. Set a P1000 pipette to 200 ul
    b. Insert the tip into the priming port
    c. Turn the wheel until the dial shows 220-230 ul (to get some liquid out of flow cell)
4. Load 800 ul of the priming mix into the flow cell, avoid air bubbles. Wait 5 min.
5. Gently lift the SpotOn sample port cover to make the SpotOn sample port accessible
6. Load 200 ul of the priming mix into the flow cell via the priming port. You see a bubble in the spotON port, that will disappear.
7. Mix the prepared library gently by pipetting up and down.
8. Add 75 µl of sample to the flow cell via the SpotOn port (drop wise).
9. Close all the ports
10. Add the light shield

Run selection:

a. Start sequencing
b. Always check the following:
c. Kit: Native Barcoding Kit V14 (SQK-NBD114.96)
d. Run options: run time 16h, 20hrs or 24hrs
e. Minimal read length: 200bp
f. Barcode on both sides, mid-read barcoding and Trim barcodes
g. Basecalling: super accuracy
h. Alignment: off
i. Output: POD5 ON
j. Q-score: 8 (7 if old flow cell)
k. Output: every 10 mins



Literature

Schuele, L., Cassidy, H., Lizarazo, E., Strutzberg-Minder, K., Schuetze, S., Loebert, S., ... & Couto, N. (2020). Assessment of viral targeted sequence capture using nanopore sequencing directly from clinical samples. Viruses, 12(12), 1358.

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