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Workflow to shed light on gram-negative bacteria transcriptomes by using direct cDNA Oxford Nanopore sequencing: from sample to data collections

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We use this protocol and it's working

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Abstract

We developed a detailed workflow for whole transcriptome RNA-Seq analysis using the Oxford Nanopore MinION sequencer to profile the pathogen's gene expression under conditions mimicking its infection cycle. By leveraging direct cDNA sequencing with the latest ONT R10 chemistry, we identified temperature-regulated genes, revealing new insights into bacterial transcriptional adaptations. Our workflow provides a reproducible and cost-effective method for bacterial transcriptomics, contributing to the study of temperature-dependent regulatory mechanisms in *Y. pestis* and other pathogens of public health importance.

Protocol materials

- Maxima H Minus First Strand cDNA Synthesis Kit **Thermo Fisher** **Catalog #**K1652
- RNaseOUT RNase Inhibitor **Invitrogen - Thermo Fisher** **Catalog #**10-777-019
- RNAse Cocktail **Thermo Fisher Scientific** **Catalog #**AM2286
- sparQ PureMag Beads **Quantabio** **Catalog #**95196-060
- DNA LoBind Tube 1.5ml **Eppendorf** **Catalog #**022431021
- Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs** **Catalog #**M0492S
- ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies** **Catalog #**SQk-NBD114.24
- Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs** **Catalog #**M0367S
- Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies** **Catalog #**FLO-MIN114
- RNAprotect Bacteria Reagent **Qiagen** **Catalog #**76506
- Trizma® hydrochloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T3253
- EDTA (0.5 M) pH 8.0 RNase-free **Thermo Fisher Scientific** **Catalog #**AM9261
- Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**L7651-1G
- MICROBExpress™ Bacterial mRNA Enrichment Kit **Thermo Fisher Scientific** **Catalog #**AM1905
- Qubit RNA BR Assay Kit **Thermo Fisher Scientific** **Catalog #**Q10211
- MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific** **Catalog #**12655512
- Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies** **Catalog #**5067
- E.coli Poly (A) Polymerase - 500 units **New England Biolabs** **Catalog #**M0276L
- Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher** **Catalog #**Q32851
- Bioanalyzer High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626
- NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs** **Catalog #**E7546L
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #**E6056S
- NucleoSpin RNA **Macherey-Nagel** **Catalog #**740955
- Invitrogen™ BSA (50 mg/ml) UltraPure™ **Fisher Scientific** **Catalog #**AM2616

1

Note

For all reagents used in this complete workflow, we recommend following the supplier recommendations in terms of storage, thawing and mixing.

1 - Sample collection

20m

2 All bacterial culture volumes were stabilized by 2 volumes of RNAprotect Bacteria Reagent.

RNAprotect Bacteria Reagent **Qiagen Catalog #**76506

Calculate the required RNAprotect Bacteria Reagent volume (**2 volumes for 1 bacterial culture volume**) and **pipet it into a tube**.

Note

Here, we harvested 5 mL of exponential growth phase culture in 10 mL of RNAprotect Bacteria Reagent.

3 **Add 1 volume of bacterial culture** to the tube. **Mix immediately** by vortexing for 00:00:05 .

4 **Incubate** for 00:05:00 at Room temperature 15-25°C .

5 5000 x g, Room temperature, 00:10:00 .

6 **Discard the supernatant**. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel.

2 - Bacterial lysis and RNA extraction

3h

7 **Prepare TE buffer** ([M] 30 millimolar (mM) Tris , [M] 1 millimolar (mM) EDTA , pH 8) containing [M] 15 mg/mL Lysozyme .

Trizma® hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T3253

EDTA (0.5 M) pH 8.0 RNase-free **Thermo Fisher Scientific Catalog #**AM9261

Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L7651-1G

8 **Add** 100 µL TE buffer containing lysozyme on the pellet.

9 **Resuspend** the pellet and **vortex** 00:00:10 each 00:02:00 during 00:10:00 at Room temperature .

10 **Add** 350 µL RA1 lysis buffer containing 3.5 µL β-mercaptoethanol and **vortex vigorously**.

NucleoSpin RNA **Macherey-Nagel Catalog #**740955

11 Reduce viscosity and **clear the lysate** by filtration through **NucleoSpin® Filter (violet ring)** in a collection tube centrifuging 11000 x g, Room temperature, 00:01:00 .

12 **Discard the NucleoSpin® Filter** and **add** 350 µL Ethanol 70% to the homogenized lysate and **mix** by pipetting up and down (5 times).

13 **Pipet lysate** up and down 2–3 times and **load** the lysate to the **NucleoSpin® RNA Column (light blue ring)** placed in a collection tube.

14 11000 x g, Room temperature, 00:00:30 and place the column in a **new Collection Tube** (2 mL).

15 **Add** 350 µL MDB (Membrane Desalting Buffer) and 11000 x g, Room temperature, 00:01:00 . **Discard flow through** and place the column back into the collection tube.

16 **Prepare DNase reaction** mixture in a sterile 1.5 mL microcentrifuge tube: For each isolation, add 10 µL reconstituted rDNase to 90 µL Reaction Buffer for rDNase. Mix by flicking the tube.

Apply 95 µL DNase reaction mixture directly **onto the center of the silica membrane** of the column.

17 **Incubate** 00:15:00 at Room temperature .

18 **Add** 200 µL Buffer RAW2 and 11000 x g, Room temperature, 00:00:30 .
Place the column into a **new Collection Tube** (2 mL).

19 **Add** 600 µL Buffer RA3 and 11000 x g, Room temperature, 00:00:30 .
Discard flow through and place the column back into the collection tube.

20 **Add** 250 µL Buffer RA3 and 11000 x g, Room temperature, 00:00:30 .
Discard flow through and place the column back into the collection tube.

21 11000 x g, Room temperature, 00:02:00 to **dry** the membrane completely.
Place the column into a 1.5 mL nuclease-free collection tube provided with the kit.

22 **Place** the 1.5 mL nuclease-free collection tube and the column with the cap opened at 50 °C for 00:02:00 to **dry** the membrane completely.

23 **Elute** the RNA in 40 µL RNase-free H2O (prewarmed at 50 °C) and **incubate** at 50 °C for 00:02:00 .

24 11000 x g, Room temperature, 00:01:00 .

25 **Recover and deposit** the eluate onto the center of the silica membrane of the column for a second elution and 11000 x g, Room temperature, 00:01:00 .

26 **Aliquot** 3 µL eluate to **evaluate the quantity and quality** of total RNA and store total RNA at -80 °C .

- **Analyze** 1 µL to evaluate the RNA concentration of total RNA .

Qubit RNA BR Assay Kit **Thermo Fisher Scientific Catalog #**Q10211

MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific** **Catalog #**12655512

Equipment	
Qubit 4	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238	LINK

- **Analyze** 1 µL to evaluate the 260/280 and 260/230 ratios.

Equipment	
NanoDrop™ 2000	NAME
Microvolume Spectrophotometer	TYPE
Thermo Scientific™	BRAND
ND-2000	SKU
https://www.thermofisher.com/order/catalog/product/fr/fr/ND-2000	LINK

- **Analyze** 1 µL to evaluate the RNA integrity number (RIN).

Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies** **Catalog #**5067

Equipment

NAME	TYPE	BRAND	SKU	LINK	SPECIFICATIONS
Bioanalyzer	Bioanalyzer	Agilent	G2991AA	https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250	Any bioanalyzer will suffice.

Expected result

Quantification obtained with the Qubit 4 Fluorometer: [M] 0.2-0.7 µg/µL
Ratios obtained with the Nanodrop: 260/280 >1.7, ideally 2.0
260/230 2.0-2.2
RIN obtained with the Bioanalyzer: >7
The RIN is calculated with the 16S and 23S rRNA peaks. The area under the 23S rRNA peak is around twice as important as the 16S one.

3 - mRNA enrichment

6h

27

MICROBExpress™ Bacterial mRNA Enrichment Kit **Thermo Fisher Scientific Catalog #**AM1905

MICROBExpress™ is designed to rapidly enrich bacterial mRNA from purified total RNA by removing the 16S and 23S ribosomal RNAs. MICROBExpress is designed so that small RNAs (including tRNA and 5S rRNA) remain in the enriched mRNA population if they were present in the total RNA treated in the procedure.

28 **Pipet** 200 µL Binding Buffer into a 1.5 mL tube provided with the kit.

29 **Add** total RNA (2-10 µg in a **maximum volume of 15 µL**) to the Binding Buffer and vortex gently to mix.

> Note
>
> For the proof of concept, we used the maximal quantity supported by the kit, 10 µg

30 **Add** 4 µL Capture Oligo Mix to the total RNA in the Binding Buffer. Vortex gently to **mix**, and microfuge briefly to get the mixture to the bottom of the tube.

31 **Heat** denaturate RNA to 70 °C for 00:10:00

32 **Anneal** at 37 °C for 00:15:00 minimum to 01:00:00 maximum (it will result in only a very modest increase in rRNA removal).

32.1 During annealing incubation, **prepare the Oligo MagBeads**.
Withdraw 50 µL Oligo MagBeads **per sample** to a 1.5 mL tube (max 500 µL per tube, i.e. max for 10 samples per tube).

> Note
>
> Record the volume of Oligo MagBeads withdrawn.

32.2 **Capture** the Oligo MagBeads by placing the tube on a magnetic stand. Leave the tube on the stand until all of the Oligo MagBeads are arranged inside the tube near the magnet.

> Note
>
> This will take 3 min or more depending the magnet stand used.

32.3 Leaving the tube on the magnet stand, carefully **remove the supernatant** by aspiration and discard the supernatant.

32.4 **Wash** the Oligo MagBeads with an **equal volume of Nuclease-free H_2O than the recorded volume** in step 32.1 by brief and gentle vortexing.

32.5 Leaving the tube on the magnet stand, carefully **remove the supernatant** by aspiration and discard the supernatant.

32.6 **Equilibrate** the Oligo MagBeads with an **equal volume of Binding Buffer** (recorded volume in step 32.1) by brief and gentle vortexing.

32.7 Leaving the tube on the magnet stand, carefully **remove the supernatant** by aspiration and discard the supernatant.

32.8 **Resuspend** the Oligo MagBeads in an **equal volume of Binding Buffer** (recorded volume in step 31.1) by gently tapping the tube.

32.9 **Prewarm** the **Oligo MagBead** slurry at 37 °C as well as the **Wash Solution** before proceeding.

33 **Add** 50 µL prewarmed prepared Oligo MagBeads to the RNA/Capture Oligo Mix (incubated at step 32).

34 **Incubate** at 37 °C for 00:15:00 .

35 **Capture** the Oligo MagBeads by placing the tube on the magnet stand.
Carefully **aspirate the supernatant** to avoid dislodging the Oligo MagBeads.
Transfer it to a Collection Tube On ice

36 **Add** 100 µL prewarmed Wash Solution to the captured Oligo MagBeads. **Resuspend the beads** by brief and gentle vortexing.

37 **Recapture** the Oligo MagBeads on the magnet stand and carefully **recover the supernatant**.
Pool this supernatant with the RNA already **in the Collection Tube** On ice .

38 **Add** 35 µL 3 M Sodium Acetate , 7 µL 5 mg/mL Glycogen , 1200 µL 100% Ethanol to the Collection Tube.

39 **Precipitate** at least at -20 °C Overnight .

Note

Prepare 70% Ethanol and store at -20 °C for the following washes.

40 15000 x g, 4°C, 00:30:00

41 Carefully **discard the supernatant** by inverting the tube. The pellet could be invisible.

42 **Wash** by adding 750 µL cold 70% Ethanol , vortex briefly.

43 15000 x g, 4°C, 00:15:00 and carefully decant and **discard the supernatant** by inverting the tube.

44 Repeat the **wash steps 42-43**

45 Short-spin the tube and carefully **remove any remaining supernatant** with a pipettor avoiding to dislodge the pellet.

46 **Air dry** the pellet for 00:05:00 maximum

47 **Resuspend** the mRNA-enriched pellet in 18 µL Nuclease-free Water and **incubate** 00:15:00 at Room temperature . If necessary, vortex the sample vigorously to resuspend the RNA.

Note

If the RNA solution is brown, there is probably a small amount of Oligo Magbeads remaining in the sample. To remove them, put the tube on the magnet stand for 3 min and move the enriched mRNA solution to a new RNase-free tube.

48 **Aliquot** 2 µL eluate to **evaluate the quantity and quality** of mRNA-enriched sample and store it at -80 °C.

- **Analyze** 1 µL to evaluate the RNA concentration.

Qubit RNA BR Assay Kit **Thermo Fisher Scientific** **Catalog #**Q10211

MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific** **Catalog #**12655512

Equipment

Qubit 4	NAME
Fluorometer	TYPE
Invitrogen	BRAND
Q33238	SKU
https://www.thermofisher.com/order/catalog/product/Q33238	LINK

- **Analyze** 1 µL to evaluate the rRNA depletion.

Bioanalyzer RNA 6000 Nano Kit **Agilent Technologies** **Catalog #**5067

Equipment

Bioanalyzer	NAME
Bioanalyzer	TYPE
Agilent	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250	LINK
Any bioanalyzer will suffice.	SPECIFICATIONS

Expected result

Quantification obtained with the Qubit 4 Fluorometer: [M] 50-200 ng/µL
RIN obtained with the Bioanalyzer: N/A
The RIN is calculated with the 16S and 23S rRNA peaks. However, the smaller the 16S and 23S rRNA peaks, the more efficient the depletion. The efficiency depends on the bacteria of interest and the starting total RNA quantity.

4 - mRNA-enriched 3'-polyadenylation

1h 30m

49 E.coli Poly (A) Polymerase - 500 units **New England Biolabs Catalog #**M0276L

DNA LoBind Tube 1.5ml **Eppendorf Catalog #**022431021

In a 1.5 ml microcentrifuge tube, **set up 3′ polyadenylation reaction** as follows:

	A	B
	Reagent	**Volume**
	Non-polyadenylated mRNA-enriched	X µl
	10X E. coli poly(A) polymerase buffer	2 µl
	ATP (10 mM)	2 µl
	Nuclease-free water	15-X µl
	E. coli poly(A) polymerase (5 U/µl)	1 µl
	TOTAL	20 µl

Note

The quantity of starting mRNA-enriched depends on the quantity obtained after rRNA depletion. We recommend using more than 300 ng and less than 10 µg. Here, we used 1,5 µg.

50 **Incubate** at 37 °C for 00:00:45 . The recommendations from Oxford Nanopore are between 0.5 and 1.5 minutes maximum.

51 **Stop the reaction** by adding 5 µL 50 mM EDTA .

52 sparQ PureMag Beads **Quantabio Catalog** #95196-060

Equilibrate at Room temperature for at least 00:30:00 **before use** and vigorously vortex to **resuspend the beads**.

Add 100 µL SparQ PureMag Beads (0.8X for RNA fragments >150 bp, decreased the ratio until 0.5X to select long fragments or increase up to 0.9X to keep the short fragments).

53 **Incubate** on a Hula Mixer 100 rpm, 00:05:00 at Room temperature .

Equipment

Rotator Mixer Multi 1	NAME
Hula mixer	TYPE
StarLab	BRAND
N2400-5000	SKU
https://www.starlabgroup.com/en/product/rotator-mixer-multi1-n2400-5000.html	LINK

54 Spin down the sample and **pellet on a magnet stand**. Keeping the tube on the magnet, pipet off and **discard the supernatant**.

55 **Wash** the beads with 200 µL 80% ethanol freshly prepared . **Vortex** 00:01:00 and **pellet** on a magnet stand. Pipet off and **discard the supernatant**.

56 **Repeat** the previous wash (step 54).

57 Briefly spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol.**
Allow to **dry** for 00:00:30 or more but do not dry the pellet to the point of cracking.

58 Remove the tube from the magnet stand and **resuspend** the pellet in 9 µL nuclease-free H2O . **Incubate** 00:05:00 at Room temperature .

59 Pellet the beads on a magnet until the eluate is clear and colourless.
Recover the eluate containing the 3′-polyadenylated RNA in a 1.5 mL Eppendorf LoBind tube.

60 **Analyze** 1 µL final eluate and quantify the final concentration using a Qubit RNA BR assay kit.
concentration

Qubit RNA BR Assay Kit **Thermo Fisher Scientific** **Catalog #**Q10211

MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific** **Catalog #**12655512

Expected result

The expected concentration of 3'-polyadenylated mRNA-enriched as a function of input material: [M] 15-100 ng/µL

61 Store the samples at -80 °C or proceed immediately with the library preparation, keeping your sample On ice .

5 - Reverse transcription and strand-switching

2h 30m

62

Note

From this point, we followed the SQK-LSK114 ONT protocol (version DCS_9187_v114_revG_19Apr2023) coupled to the SQK-NBD114.24 protocol (version NBA_9168_v114_revL_15Sep2022) with few adjustments.
It is important to check the newly available protocol update on the Oxford Nanopore Community to take notes about the potential new major recommendations.

Transfer 100 ng of polyA mRNA into 0.2 mL PCR tube and **adjust the volume to 7.5 µL** with nuclease-free H_2O.

63 **Mix** by flicking the tube to avoid unwanted shearing and spin down briefly.

64 **Add** 2.5 µL 2 µM VN primer and 1 µL 10 mM dNTPs to the PCR tube.

Note

VN primer sequence (HPLC grade):
/5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTVN

65 **Mix** by flicking the tube to avoid unwanted shearing and spin down briefly.

66 **Incubate** at 65 °C for 00:05:00 .

67 **Snap cool** On ice or on a prechilled freezer block for 00:01:00 .

68 Meanwhile, **prepare the following mix** in a separate tube:

	A	B
	Reagent	**Volume per sample**
	5X RT Buffer	4 µL
	RNaseOUT	1 µL
	Nuclease-free Water	1 µL
	10 µM Strand-Switching primer	2 µL

Mix gently by flicking the tube and spin down.

RNaseOUT RNase Inhibitor **Invitrogen - Thermo Fisher Catalog #**10-777-019

Maxima H Minus First Strand cDNA Synthesis Kit **Thermo Fisher Catalog #**K1652

Note

Strand-Switching primer sequence (HPLC grade):
TTTCTGTTGGTGCTGATATTGCTmGmGmG
(mG = 2' O-Methyl RNA bases)

69 **Add** 8 µL Strand-Switching mix to the snap-cooled mRNA. **Mix** by flicking the tube and spin down.

70 **Incubate** in a thermal cycler at 42 °C 00:02:00

71 **Add** 1 µL Maxima H minus Reverse Transcriptase . **Mix** by flicking the tube and spin down.

72 **Incubate** using the following program in a thermal cycler:
- Reverse transcription and strand switching 42 °C 01:30:00
- Heat inactivation 85 °C 00:05:00
- Hold 4 °C

6 - RNA degradation and second strand synthesis 2h

73 **Add** 1 µL RNAse Cocktail to the reverse transcription reaction.

RNAse Cocktail **Thermo Fisher Scientific Catalog #**AM2286

74 Incubate at 37 °C for 00:10:00 in a thermal cycler.

75 Meanwhile, **resuspend the SparQ PureMag Beads** by vortexing.
Add 17 µL SparQ PureMag Beads in a 1.5 mL Eppendorf LoBind tube.

sparQ PureMag Beads **Quantabio Catalog #**95196-060
DNA LoBind Tube 1.5ml **Eppendorf Catalog #**022431021

76 **Transfer the sample to the beads** and mix by flicking the tube.

77 **Incubate** on a Hula mixer 100 rpm, 00:05:00 at Room temperature .

78 Spin down the sample and **pellet** on a magnet stand. **Pipet off the supernatant**.

79 **Wash** the beads with 200 µL fresh 80 % Ethanol without disturbing the pellet.
Discard the ethanol using a pipette.

80 **Repeat** the previous step.

81 Spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol**.
Allow to **dry** for 00:00:30 , but do not dry the pellet to the point of cracking.

82 Remove the tube from the magnet stand and **resuspend the pellet** in 10 µL Nuclease-free H2O .

83 **Incubate** on a Hula mixer 100 rpm, 00:10:00 at Room temperature .

84 Briefly spin down the tube and **pellet** the beads **on the magnet stand** until the eluate is clear and colourless, for at least 00:01:00 .

85 Remove and **retain** 10 µL eluate into a 0.2 ml PCR tube.

86 **Prepare the following reaction mix** in a 1.5 ml tube:

	A	B
	Reagent	**Volume per sample**
	Q5 High-Fidelity 2X Master Mix	12.5 µL
	10 µM PR2 primer	1 µL
	Nuclease-free H2O	1.5 µL

Mix gently by flicking the tube and spin down.

Q5 High-Fidelity 2X Master Mix - 100 rxns **New England Biolabs Catalog #**M0492S

Note

PR2 primer sequence (HPLC grade):
/5Phos/TTTCTGTTGGTGCTGATATTGC

87 **Add** 15 µL reaction mix to the reverse-transcribed sample in the PCR tube.

88 **Incubate** using the following program in a thermal cycler:

- Denaturation 94 °C 00:01:00

- Annealing 50 °C 00:01:00
- Extension 72 °C 00:15:00
- Hold 4 °C

89 Meanwhile, **resuspend the SparQ PureMag Beads** by vortexing.
Add 20 µL SparQ PureMag Beads in a 1.5 mL Eppendorf LoBind tube.

sparQ PureMag Beads **Quantabio Catalog #**95196-060
DNA LoBind Tube 1.5ml **Eppendorf Catalog #**022431021

90 **Transfer the sample to the beads** and **mix** by flicking the tube.

91 **Incubate** on a Hula mixer 100 rpm, 00:05:00 at Room temperature .

92 Spin down the sample and **pellet on a magnet stand**. **Pipet off the supernatant**.

93 **Wash** the beads with 200 µL fresh 80 % Ethanol without disturbing the pellet.
Discard the ethanol using a pipette.

94 **Repeat** the previous step.

95 Spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol**.
Allow to **dry** for 00:00:30 , but do not dry the pellet to the point of cracking.

96 Remove the tube from the magnet stand and **resuspend** the pellet in
14 µL Nuclease-free H2O .

97 **Incubate** on a Hula mixer 100 rpm, 00:10:00 at Room temperature .

98 Briefly spin down the tube and **pellet the beads on the magnet stand** until the eluate is clear and colorless, for at least 00:01:00 .

99 Remove and **retain** 14 µL eluate into a 1.5 mL Eppendorf LoBind tube.

100 **Analyze** 1 µL strand-switched cDNA with a Qubit fluorometer using the Qubit dsDNA HS assay kit.

Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851
MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific Catalog #**12655512

Expected result

The expected concentration of cDNA: [M] 0.5-2 ng/µL

101 To calculate the cDNA molarity, analyze 1 µl of the strand-switched cDNA length with a Bioanalyzer.

Note

If the mean cDNA length is known, the molarity could be calculated with the NEBioCalculator.

102 Store the samples at -20 °C for long-term storage or at 4 °C for short-term storage.

7 - End-prep

1h 30m

103 ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies Catalog #**SQk-NBD114.24

The ONT Native barcoding sequencing kit v14 (24) contains 24 barcodes. In other words, the maximum number of samples to be simultaneously run in compatible R10.4.1 flow cells is 24.

104 **Dilute the DNA Control Sample** (DCS) by adding 105 µL Elution Buffer (EB) **directly to one DCS tube**. The **final concentration is 10 ng/µL**.
Mix gently by pipetting and spin down.

105 In 0.2 ml PCR tubes, **aliquot the same highest quantity possible of cDNA per sample up to 11.5 µL**.

Note

Expect a minimum of 70 fmol for all samples summed. To date, we have not go below 5 fmol of cDNA per sample with 24 samples. Here, we used 14 fmol of cDNA per sample.

106 **Make up each sample to 11.5 µl** using Nuclease-free H2O. **Mix** gently by pipetting and spin down.

107 **Calculate the DNA Control Sample volume** (X µL) to put in each cDNA sample. To know, 1 µL of DCS should be used for 200 fmol (130 ng for 1 kb cDNA, the maximum quantity per sample) of cDNA sample.

Note

Here, for 14 fmol of cDNA, we used 0.07 µL DCS per sample.

Prepare the following end-prep mix:

	A	B
	Reagent	**Volume per sample**
	DNA Control Sample	X µL
	Ultra II End-prep Reaction Buffer	1.75 µL
	Ultra II End-prep Enzyme Mix	0.75 µL
	Nuclease-free H2O	1 - X µL

Mix gently by flicking the tube and spin down.

NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog** #E7546L

108 **Add** 3.5 µL end-prep mix to the 11.5 µL of cDNA sample. **Mix** by pipetting and spin down.

109 **Incubate** in a thermal cycler 20 °C 00:05:00 and 65 °C 00:05:00 .

110 Meanwhile, by vortexing, **resuspend** the room-temperature equilibrated **AMPure XP beads (AXP)** provided in the ONT kit and **add** 15 µL AXP into as many 1.5 ml Eppendorf DNA LoBind tubes as samples.

111 **Transfer each cDNA sample** into the 1.5 ml Eppendorf DNA LoBind tubes containing AXP.
Mix by flicking the tube.

112 **Incubate** on a Hula mixer for 00:05:00 at Room temperature .

113 Spin down the sample and **pellet** on a magnet stand until the eluate is clear and colorless. **Pipet off the supernatant**.

114 **Wash** the beads with 200 µL fresh 80 % Ethanol without disturbing the pellet.
Discard the ethanol using a pipette.

115 **Repeat** the previous step.

116 Spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol**.
Allow to **dry** for 00:00:30 , but do not dry the pellet to the point of cracking.

117 Remove the tube from the magnet stand and **resuspend** the pellet in 9 µL Nuclease-free H2O .

118 **Incubate** on a Hula mixer 100 rpm, 00:02:00 at Room temperature .

119 Briefly spin down the tube and **pellet the beads on the magnet stand** until the eluate is clear and colorless, for at least 00:01:00 .

120 Remove and **retain** 9 µL eluate into a 1.5 mL Eppendorf LoBind tube.

121 **Analyze** 1 µL end-prepped cDNA with a Qubit fluorometer using the Qubit dsDNA HS assay kit.

Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851

MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific Catalog #**12655512

Expected result

The expected concentration of end-prepped cDNA: [M] 1-4 ng/µL

122 **Take forward the highest equimolar mass possible of each sample to be barcoded up to 7.5 µL** into the native barcode ligation step. However, you may store the samples at 4°C overnight.

Note

To date, we fruitfully tested the range 7-30 ng cDNA per sample.
Here, we pooled 15 ng (16 fmol) of each end-prepped cDNA sample.

8 - Native barcode ligation

2h

123 ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies Catalog #**SQk-NBD114.24

Select a unique barcode for each sample to be run together on the same flow cell.
Up to 24 samples can be barcoded and combined in one experiment.

124 In 0.2 ml PCR tubes, **add the reagents in the following order**:

	A	B
	Reagent	**Volume**
	End-prepped DNA	7.5 µL
	Native Barcode (NB01-24)	2.5 µL
	Blunt/TA Ligase Master Mix	10 µL

Thoroughly **mix** the reaction by gently pipetting and briefly spinning down

Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog #**M0367S

125 **Incubate** for 00:20:00 at Room temperature .

126 The following volume of EDTA depends on the cap color EDTA tube in the ONT kit.
Add 2 µL clear cap EDTA or 4 µL blue cap EDTA to each tube, **mix** thoroughly by pipetting and spin down briefly.

127 **Pool all the barcoded** samples in a 1.5 mL Eppendorf DNA LoBind tube.

128 By vortexing, **resuspend** the room-temperature equilibrated **AMPure XP beads (AXP)** provided.

129 **Add AMPure XP Beads (AXP)** to the pooled reaction, and mix by pipetting for a **0.4X clean**.
In other words: 14.67 µL AXP per sample used if you added the clear cap EDTA
OR 16 µL AXP per sample used if you added the blue cap EDTA

Note

Here, we added blue cap EDTA in 20 barcoded samples, thus we performed a 320 µL AXP clean.

130 **Incubate** on a Hula mixer 100 rpm, 00:10:00 at Room temperature .

131 Spin down the sample and **pellet on a magnet stand** for 00:05:00 until the eluate is clear and colorless. **Pipet off the supernatant**.

132 Keeping the tube on the magnet stand, **wash** the beads with 700 µL fresh 80% Ethanol without disturbing the pellet. **Discard the ethanol** using a pipette.

133 **Repeat** the previous step.

134 Spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol**. Allow to **dry** for 00:00:30 , but do not dry the pellet to the point of cracking.

135 Remove the tube from the magnet stand and **resuspend** the pellet in 33 µL Nuclease-free H2O by gently flicking the tube.

136 **Incubate** for 00:10:00 at 37 °C . **Agitate** the sample for 00:00:10 by gently flicking the tube **every** 00:02:00 to encourage DNA elution.

137 **Pellet the beads on a magnet stand** until the eluate is clear and colorless.

138 Remove and **retain** 33 µL eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

139 **Analyze** 1 µL barcoded cDNA with a Qubit fluorometer using the Qubit dsDNA HS assay kit.

Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851

MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific Catalog #**12655512

Expected result

The expected concentration of pooled barcoded sample: [M] 3-10 ng/µL

140 Take forward the barcoded DNA library to the adapter ligation step. However, you may store the sample at 4°C overnight.

9 - Adapter ligation 2h

141 ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies Catalog #**SQk-NBD114.24

In a 0.2 mL PCR tube, **mix in the following order**:

	A	B
	Reagent	**Volume**
	barcoded DNA library	30 µL
	Native Adapter	5 µL
	NEBNext Quick Ligation Reaction Buffer (5X)	10 µL
	Quick T4 DNA Ligase	5 µL

Thoroughly mix the reaction by gently pipetting and briefly spinning down.

NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S

142 **Incubate** the reaction for 00:20:00 at Room temperature in a thermal cycler.

143 **Resuspend** by vortexing the room-temperature equilibrated **AMPure XP beads** (AXP) provided.

144 **Transfer** into a clean 1.5 mL Eppendorf DNA LoBind tube and **add** 20 µL AXP to the reaction and **mix** by pipetting.

145 **Incubate** on a Hula mixer 100 rpm, 00:10:00 at Room temperature .

146 Spin down the sample and **pellet on a magnet stand** until the eluate is clear and colorless. **Pipet off the supernatant**.

147 **Wash** the beads by adding 125 µL Short Fragment Buffer (SFB) to retain DNA fragments of all sizes.

148 **Flick the beads** to resuspend and spin down.
Return the tube to the magnet stand and allow the beads to pellet.
Discard the supernatant using a pipette.

149 **Repeat** the previous wash.

150 Spin down and place the tube back on the magnet stand. **Pipet off any residual ethanol**.

151 Remove the tube from the magnetic rack and **resuspend** the pellet in 15 µL Elution Buffer (EB) .

152 Spin down and **incubate** for 00:10:00 at 37 °C . **Agitate** the sample for 00:00:10 by gently flicking **every** 00:02:00 to encourage DNA elution.

153 **Pellet the beads on a magnet stand** until the eluate is clear and colorless, for at least 00:01:00 .

154 Remove and **retain** 15 µL eluate into a clean 1.5 mL Eppendorf DNA LoBind tube.

155
- **Analyze** 1 µL adapted DNA library with a Qubit fluorometer using the Qubit dsDNA HS assay kit.

Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851
MICROTUBE 0.5 ML PP NEUTRAL **Fisher Scientific Catalog #**12655512

- **Analyze** 1 µL adapted DNA library with a Bioanalyzer to evaluate the length distribution of the library and to calculate the molarity.

Bioanalyzer High Sensitivity DNA Kit **Agilent Technologies Catalog #**5067-4626

Equipment

Bioanalyzer	NAME
Bioanalyzer	TYPE
Agilent	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/bioanalyzer-automated-electrophoresis/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250	LINK
Any bioanalyzer will suffice.	SPECIFICATIONS

Expected result

The expected concentrations of adapted DNA library: [M] 3-10 ng/µL [M] 3-20 nanomolar (nM)

156 **Make up the library to** 12 µL containing 35-50 fmol (for 1-10kb library length) or 100 fmol (for <1kb library length) .

The prepared library is ready for loading onto the R10.4.1 flow cell. **Store the library on ice until loading**.

Note

Here, we loaded around 100 fmol.

10 - Priming and loading the SpotON flow cell 45m

157 On the computer, **plug in the MinION**, **check** for the operating system **updates** and prohibit it during the run. **Get the last MinKNOW software version** and **start a hardware check**.

Software

MinKNOW™ NAME

Oxford Nanopore DEVELOPER

https://community.nanoporetech.com/technical_documents/minknow-tech-doc/v/mitd_5000_v1_revaj_16may2016/introduction-to-the-minkno SOURCE LINK

Note

We recommend installing the GPU version instead of the CPU if your computer complies with the minimum ONT requirements to avoid run crash.

158 **Open** the MinION device **lid** and **slide the R10.4.1 flow cell** under the clip. Press down firmly on the flow
cell to ensure correct thermal and electrical contact. **Start a flow cell check**.

Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114

Note

ONT will replace any flow cell with fewer than **800 nanopores** when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. The warranty on this product is 12 weeks from receipt by the customer.

159 Meanwhile, at Room temperature, **prepare the flow cell priming mix** as directed below:

	A	B
	Reagent	**Volume**
	Flow Cell Flush (FCF)	1170 µL
	50 mg/ml Bovine Serum Albumin (BSA)	5 µL
	Flow Cell Tether (FCT)	30 µL

Mix by pipetting.

ONT Native barcoding sequencing kit v14 (24) **Oxford Nanopore Technologies Catalog #**SQk-NBD114.24

Invitrogen™ BSA (50 mg/ml) UltraPure™ **Fisher Scientific Catalog #**AM2616

160 Slide the flow cell priming port cover clockwise to **open the priming port** and draw back a small volume to
remove any bubbles in the following way:
1) Set a P1000 pipette to 200 µl
2) Insert the tip into the priming port
3) Turn the wheel until you can see a small volume of yellow buffer entering the pipette tip

Note

Visually check that there is continuous buffer from the priming port to the beginning of the waste channel after the sensor array to be sure that the array of pores is covered by buffer at all times.

161 Avoiding the introduction of air bubbles, **load** 800 µL priming mix into the flow cell via the **opened priming port** (form a drop at the end of the pipette tip before inserting into the priming port and gently load the priming mix). You can see the yellow buffer enter the waste channel.

162 **Wait** for 00:05:00 .

163 During this time, in a 1.5 ml Eppendorf DNA LoBind tube, **prepare the library as follows**:

	A	B
	Reagent	**Volume**
	Sequencing Buffer (SB)	37.5 µL
	(Pipet mix immediately before use) Library Beads (LIB)	25.5 µL
	adapted cDNA library	12 µL

164 Gently **lift the SpotON sample port cover** (to make the SpotON sample port accessible and enable the creation of a drop before sample loading).

165 To complete the flow cell priming, **load** 200 µL priming mix into the flow cell via the **opened priming port** (not the SpotON sample port), avoiding the introduction of air bubbles as already mentioned.

166 **Mix** the prepared library gently by pipetting and immediately **load** 75 µL prepared library to the flow cell via the **SpotON sample port** in a **dropwise fashion**. Ensure each drop flows into the port before adding the next.

167 Gently **replace the SpotON sample port cover**, making sure the bung enters the SpotON port and **close the priming port**.

168 **Close the device lid** and **set up a sequencing run** on MinKNOW.

Fill in the fields step-by-step (Experiment ID, Sample ID) and select the kit SQK-NBD114.24.
In sequencing analysis settings, basecalling and barcoding should be ON, select Trim barcodes since each barcode data is separately stored, turn the alignment ON and check the output settings. Make sure there is enough free disk space (at least 500 GB) and use POD5 file extension to rebasecall. The MinQ Score should be 8. To determine the sequencing duration, choose 72 h or less, or a number of reads depending on the objectives.

Note

We recommend downloading the sequence FASTA file of your strain to benefit from the real-time analysis in terms of sequence coverage. In this way, you can stop the run when the required sequencing depth is obtained (10X minimum for transcriptomes).