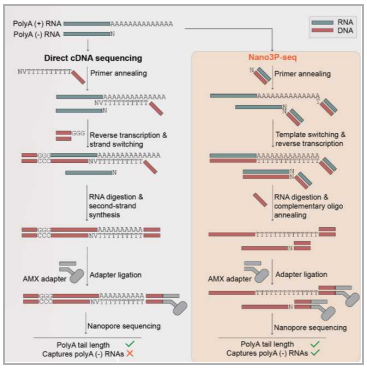

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Protocol status: In development
We are still developing and optimizing this protocol

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Nano3P-seq Protocol V.3

Oguzhan Begik[1]

[1]Centre for Genomic Regulation

Eva Maria Novoa
Center for Genomic Regulation (CRG)

DISCLAIMER

This is the modified protocol to use the LSK114 kit instead of deprecated DCS109. Results generated in the manuscript were using DCS109. We do not guarantee that all results will be the same with change of this kit, extensive/systematic benchmarking has not been performed with this change of kit.

ABSTRACT

NOTE: in this protocol version, we use LSK114 kit, replacing deprecated DCS109 kit

Here, we develop Nanopore 3’ end-capture sequencing (Nano3P-seq), a novel method that relies on nanopore cDNA sequencing to simultaneously quantify RNA abundance, tail composition and tail length dynamics at per-read resolution. By employing a template switching-based sequencing protocol, Nano3P-seq can sequence any given RNA molecule from its 3’ end, regardless of its polyadenylation status, without the need for PCR amplification or ligation of RNA adapters.

Without Barcoding

1

Materials and consumables required

●Ligation Sequencing Kit v14 (ONT, SQK-LSK114)
●AMPure XP Reagent (Agencourt , A63881)
●TGIRT™-III Enzyme (InGex)
●RNase Inhibitor, Murine (NEB,M0314L)
●RNase Cocktail Enzyme Mix (ThermoFisher,AM2286)
●Qubit dsDNA HS Assay Kit (ThermoFisher, Q32854)
●10 mM dNTP solution (ThermoFisher, R0191)
●0.1 M DTT solution (ThermoFisher, 707265ML)
●1.5 mL Eppendorf DNA LoBind Tubes (Eppendorf, 0030108051)
●0.2 ml thin-walled PCR tubes (Starlab, A1402-3700)
●1 M Nuclease-free Tris-Cl pH 7.5 (ThermoFisher, 15567027)
●5 M Nuclease-free NaCl (ThermoFisher, AM9760G)
●1 M Nuclease-free MgCl2(ThermoFisher,AM9530G)
●Nuclease-free water (ThermoFisher, AM9922)
●Freshly prepared 70% ethanol in nuclease-free water

Oligos required

	A	B
	Oligo Name	Sequence
	D_DNA (Standard desalting)	/5Phos/CTTCCGATCACTTGCCTGTCGCTCTATCTTCN
	R_RNA (RNase-Free HPLC)	rGrArArGrArUrArGrArGrCrGrArCrArGrGrCrArArGrUrGrArUrCrGrGrArArG/3SpC3/
	CompA_DNA (Standard desalting)	GAAGATAGAGCGACAGGCAAGTGATCGGAAGA

Oligos required for the Nano3P-seq library

1.1 Preannealing of the oligos

We need pre-anneal R_RNA and D_DNA oligos in order to be able to initiate template switching.

	A	B	C	D
	Reagent	Initial Concentration	Volume	Final Conc
	R_RNA	10 uM	1 uL	1 uM
	D_DNA	10 uM	1 uL	1 uM
	Tris-Cl pH 7.5	0.1 M	1 uL	0.01 M
	NaCl	0.5 M	1 uL	0.05 M
	RNase Inhibitor, Murine		0.5 uL	
	dH2O		5 uL	
	Total		10 uL	

● Heat the mixture for 94°C for 1 mins and ramp down to 25°C at 0.1°C/s (in thermal cycler).

1.2 Reverse Transcription

	A	B	C	D
	Component	Initial Conc	Volume	Final Conc/Amount
	5X Reaction Buffer	2.25 M NaCl, 25 mM MgCl2, 100 mM Tris-HCl, pH 7.5	4 uL	450 mM NaCl, 5 mM MgCl2, 20 mM Tris-HCl, pH 7.5
	DTT	0.1 M	1 uL	5 mM
	Pre-annealed oligos	1 uM	2 uL	0.1 uM
	RNA		Up to 10 uL	50-100 ng
	TGIRT	10uM- 200 Unit/ul	1-2 uL	500 nM- 1000 nM
	RNase Inhibitor, Murine		1 uL	
	Total		19 uL	

● *Pre-incubate at room temperature for 30 minutes, then add **1.25 ul of 10 mM dNTPs***
● Incubate at 60°C for 1 hour
● Inactivate the enzyme by incubating at 75°C for 15 mins
● Move reaction to ice

RNase treatment

● Add 1.5 ul RNase Cocktail Enzyme Mix to each tube

● 37°C 10 minutes incubation
● Move reaction to ice

Cleanup using Ampure XP Beads

● Mix the samples with the appropriate volume of beads (17 ul, 0.8 X keeps everything above 150 nt, good for getting rid of adapters)
● Mix the beads by flicking
● Incubate 10 minutes at room temperature
● Spin down the tube and place it on the magnet
● Remove the supernatant
● Add 70% freshly prepared 200 ul ethanol to the tube
● Incubate for 30 seconds at room temperature
● Remove the ethanol completely by spinning down the tube, placing back it on magnet and removing the liquid
● Air-dry the pellet for maximum 1 minute, do not let it dry out completely!
● Resuspend the beads in 16 ul water
● Incubate5-10 minutes in RT
● Place the beads on magnet
● Transfer the supernatant into a new tube.
● Quantify 1 µl of eluted sample using a Qubit fluorometer
● Move to the next step

1.3 Annealing of Complementary DNA to VNP Oligo

This step is essential to have a double-stranded DNA oligo with an A overhang, which will initiate the ligation to the adapter

	A	B	C	D
	Components	Initial Concentration	Final Conc.	Volume
	cDNA			15 uL
	Tris-Cl pH 7.5	0.1 M	0.01 M	2.25 uL
	NaCl	0.5 M	0.05 M	2.25 uL
	CompA_DNA	10 uM	0.44 uM	1 uL
	Water			2 uL
	Total			22.5 uL

- Mix by flicking
- Heat the mixture for 90°C for 1 mins and ramp down to RT at 0.1°C/s (in thermalcycler).
- Mix the following

1.4 Ligation Adapter (LA) ligation

22.5 uL cDNA-complement mix
37.5 uL dH2O
25 ul Ligation Buffer (LNB) from SQK-LSK114
5 uL Ligation Adapter (LA) from SQK-LSK114
10 uL NEBNext Quick T4 DNA Ligase (Concentrated T4 DNA Ligase)

- Mix by flicking or pipetting slowly
- Spin down
- Incubate at RT for 10 minutes

Ampure XP Beads Cleanup

- Add 50 ul resuspended AMPure XP beads (0.5X) to the reaction and mix by flicking
- Incubate 10 minutes at room temperature
- Thaw Short Fragment Buffer (SFB) and Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice. Check if the contents of each tube are clear of any precipitate. (SFB is used to keep all the fragments)
- Spin down the tube and place it on the magnet
- Remove the supernatant
- Add 250 ul SFB to the beads. Close the tube lid, and resuspend the beads by flicking. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant
- Repeat the previous step.
- Remove the SFB completely by spinning down the tube, pipetting out the liquid and placing back it on magnet
- Air-dry the pellet for maximum 1 minute, do not let it dry out completely!
- Resuspend the beads in 15 ul Elution Buffer (EB)
- Incubate 5-10 minutes in RT
- Place the beads on magnet
- Place the elute in a 1.5 ml tube
- Quantify 1 µl of eluted sample using a Qubit fluorometer

Make up the library to 12 ul at 10-20 fmol. It is recommended to load 10-20fmol for optimal sequencing results. Calculate fmol amount using NEBiocalculator based on your expected library size.

- Move to the “Prepare the library for loading” step.

2 Materials and consumables required

●Ligation Sequencing Kit v14 (ONT, SQK-LSK114)
●Native Barcoding Kit 24 V14 (ONT, SQK-NBD114.24)
● NEBNext Quick Ligation Module (E6056)
● Blunt/TA Ligase Master Mix (NEB, M0367)
●AMPure XP Reagent (Agencourt , A63881)
●TGIRT™-III Enzyme (InGex)
●RNase Inhibitor, Murine (NEB,M0314L)
●RNase Cocktail Enzyme Mix (ThermoFisher,AM2286)
●Qubit dsDNA HS Assay Kit (ThermoFisher, Q32854)
●10 mM dNTP solution (ThermoFisher, R0191)
●0.1 M DTT solution (ThermoFisher, 707265ML)
●1.5 mL Eppendorf DNA LoBind Tubes (Eppendorf, 0030108051)
●0.2 ml thin-walled PCR tubes (Starlab, A1402-3700)
●1 M Nuclease-free Tris-Cl pH 7.5 (ThermoFisher, 15567027)
●5 M Nuclease-free NaCl (ThermoFisher, AM9760G)
●1 M Nuclease-free MgCl2(ThermoFisher,AM9530G)
●Nuclease-free water (ThermoFisher, AM9922)
●Freshly prepared 70% ethanol in nuclease-free water

Oligos required

	A	B
	Oligo Name	Sequence
	D_DNA (Standard desalting)	/5Phos/CTTCCGATCACTTGCCTGTCGCTCTATCTTCN
	R_RNA (RNase Free HPLC)	rGrArArGrArUrArGrArGrCrGrArCrArGrGrCrArArGrUrGrArUrCrGrGrArArG/3SpC3/
	CompA_DNA (Standard desalting)	GAAGATAGAGCGACAGGCAAGTGATCGGAAGA

2.1 Preannealing of the oligos

We need pre-anneal R_RNA and D_DNA oligos in order to be able to initiate template switching.

	A	B	C	D
	Reagent	Initial Concentration	Volume	Final Conc
	R_RNA	10 uM	1 uL	1 uM
	D_DNA	10 uM	1 uL	1 uM
	Tris-Cl pH 7.5	0.1 M	1 uL	0.01 M
	NaCl	0.5 M	1 uL	0.05 M
	RNase Inhibitor, Murine		0.5 uL	
	dH2O		5 uL	
	Total		10 uL	

●Heat the mixture for 94°C for 1 mins and ramp down to 25°C at 0.1°C/s (in thermal cycler).

2.2 Reverse Transcription

Important note: Prepare one reaction for each sample/barcode

	A	B	C	D
	Component	Initial Conc	Volume	Final Conc/Amount
	5X Reaction Buffer	2.25 M NaCl, 25 mM MgCl2, 100 mM Tris-HCl, pH 7.5	4 uL	450 mM NaCl, 5 mM MgCl2, 20 mM Tris-HCl, pH 7.5
	DTT	0.1 M	1 uL	5 mM
	Pre-annealed oligos	1 uM	2 uL	0.1 uM
	RNA		Up to 10 uL	50-100 ng
	TGIRT	10uM- 200 Unit/ul	1-2 uL	500 nM- 1000 nM
	RNase Inhibitor, Murine		1 uL	
	Total		19 uL	

- *Pre-incubate at room temperature for 30 minutes, then add **1 .25 ul of 10 mM dNTPs***
- Incubate at 60°Cfor 1 hour
- Inactivate the enzyme by incubating at 75°C for 15 mins
- Move reaction to ice

RNase treatment

● Add 1.5 ul RNase Cocktail Enzyme Mix to each tube
● 37°C 10 minutes incubation
● Move reaction to ice

Cleanup using Ampure XP Beads

● Mix the samples with 17 ul of beads (0.8X, depends on the size of your library, please refer to size selection by Ampure XP beads)
● Mix the beads by flicking
● Incubate 10 minutes at room temperature
● Spin down the tube and place it on the magnet
● Remove the supernatant
● Add 70% freshly prepared 200 ul ethanol to the tube
● Incubate for 30 seconds at room temperature
● Remove the ethanol
● Repeat the washing
● Remove the ethanol completely by spinning down the tube, placing back it on magnet and removing the liquid
● Air-dry the pellet for maximum 1 minute, do not let it dry out completely!
● Resuspend the beads in 6 ul water
● Incubate 5-10 minutes in RT
● Place the beads on magnet
● Transfer the supernatant into a new tube.
● Quantify 1 µl of eluted sample using a Qubit fluorometer

2.3 Annealing of Complementary DNA to VNP Oligo

This step is essential to have a double-stranded DNA oligo with an A overhang, which will initiate the ligation to the adapter

	A	B	C	D
	Components	Initial Concentration	Final Conc.	Volume
	cDNA			5 uL
	Tris-Cl pH 7.5	0.1 M	0.01 M	0.75 uL
	NaCl	0.5 M	0.05 M	0.75 uL
	CompA_DNA	3 uM	0.4 uM	1 uL
	Total			7.5 uL

● Mix by flicking

● Heat the mixture for 90°C for 1 mins and ramp down to RT at 0.1°C/s (in thermalcycler).
● Mix the following

2.4 Native Barcode Ligation

	Reagent	Volume
	Complementary oligo - cDNA	7.5 µl
	Native Barcode (NB01-24)	2.5 µl
	Blunt/TA Ligase Master Mix	10 µl
	Total	**20 µl**

● Mix by flicking or gently pipetting
● Spin down
● Incubate at RT for 20 minutes
● Add 2 µl of EDTA to each well and mix thoroughly by pipetting and *spin down* briefly.
● At this step, we inactivate the ligation reaction.
Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

	A	Volume per sample	For 6 samples	For 12 samples	For 24 samples
	Total volume	22 µl	132 µl	264 µl	528 µl

Cleanup using Ampure XP Beads
●Mix the samples with 0.5X volume of beads (0.5X, depends on the size of your library, please refer to size selection by Ampure XP beads)
●Mix the beads by flicking
●Incubate 10 minutes at room temperature
●Spin down the tube and place it on the magnet
●Remove the supernatant
●Add 80% freshly prepared 700 ul ethanol to the tube
●Incubate for 30 seconds at room temperature
●Remove the ethanol
●Repeat the washing
●Remove the ethanol completely by spinning down the tube, placing back it on magnet and removing the liquid
●Air-dry the pellet for maximum 1 minute, do not let it dry out completely!
●Resuspend the beads in 31 ul water
●Incubate5-10 minutes in RT
●Place the beads on magnet

●Transfer the supernatant into a new tube.
●Quantify 1 µl of eluted sample using a Qubit fluorometer

Note: Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.
To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)
To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

Native Adapter (NA) Ligation
In a 1.5 ml Eppendorf LoBind tube, mix in the following order:

	Reagent	Volume
	Pooled barcoded sample	30 µl
	Native Adapter (NA)	5 µl
	NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
	Quick T4 DNA Ligase	5 µl
	Total	**50 µl**

●Mix gently by flicking the tube, and spin down.
●Incubate the reaction for 20 minutes at RT.

Ampure XP Beads Cleanup

●Add 25 ul resuspended AMPure XP beads (0.5X, depending on the size of library) to the reaction and mix by flicking
●Incubate 10 minutes at room temperature
●Thaw Long or Short Fragment Buffer (LFB, SFB) and Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice. Check if the contents of each tube are clear of any precipitate.
●Spin down the tube and place it on the magnet
●Remove the supernatant
●Add 125 ul SFB or LFB to the beads. Close the tube lid, and resuspend the beads by flicking. Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant
●Repeat the previous step.
●Remove the SFB/LFB completely by spinning down the tube, pipetting out the liquid and placing back it on magnet

●Air-dry the pellet for maximum 1 minute, do not let it dry out completely!
●Resuspend the beads in 15 ul Elution Buffer (EB)
●Incubate 5-10 minutes in RT
●Place the beads on magnet
●Place the elute in a 1.5 ml tube
●Quantify 1 µl of eluted sample using a Qubit fluorometer

Make up the library to 12 µl at 10-20 fmol.
We recommend loading 10 - 20 fmol of this final prepared library onto the R10.4.1 flow cell.
Loading more than 20 fmol of DNA can reduce the rate of duplex read capture.

●The prepared library is used for loading into the MinION flow cell. Store the library on ice until ready to load.
●Move to the “Prepare the library for loading” step.

Prepare the library for loading

3 **Prepare the library for loading**

TIP
Library storage recommendations We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short-term** storage or repeated use, for example, re-loading flow cells between washes.
For single use and **long-term storage** of more than 3 months, we recommend storing libraries at **-80°C** in Eppendorf DNA LoBind tubes.

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-MIN114).

Before starting:

● Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at RT. Mix by vortexing, spin down and return to ice.
- For optimal sequencing performance and improved output on MinION R10.4.1 flow cells (FLO-MIN114), we recommend adding Bovine Serum Albumin (BSA) to the flow cel priming mix at a final concentration of 0.2 mg/ml.

● Mix the contents of LIB (Library beads) with a large volume of pipette to make it homogeneous

● To prepare the flow cell priming mix with BSA, add the following directly to the tube of Flow cell Flush and mix by pipetting:

- 1,170 µl FCF
- 5 ul BSA at 50 mg/ml
- 30 ul FCT

● In the new tube, prepare the following:

- 37.5 µl Sequencing Buffer (B)
- 25.5 µl Loading Beads (LB), mixed immediately before use
- 12 µl DNA library

Priming the flow cell

QC the flowcell:

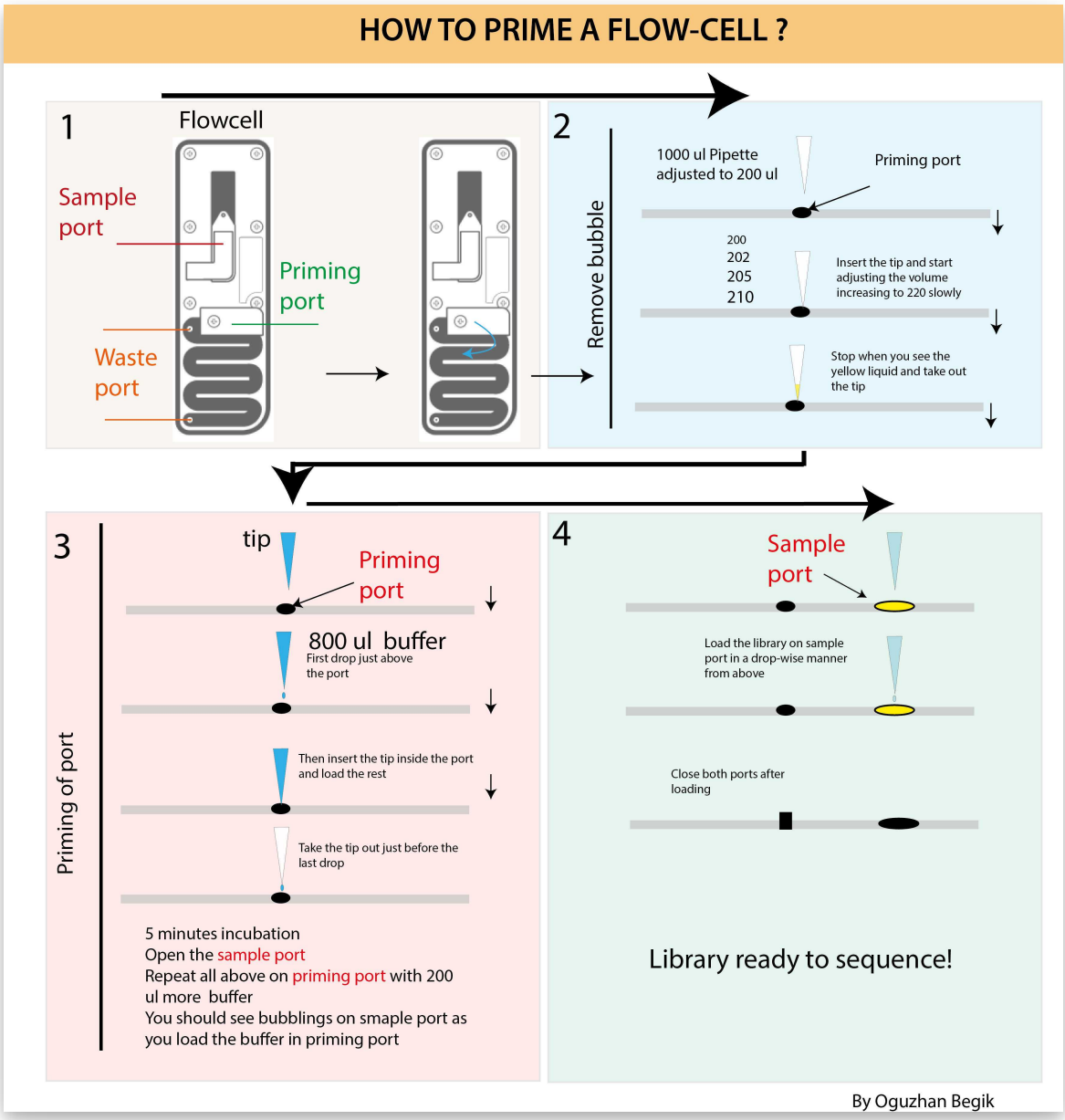
- Take the flowcell out of the fridge.Connect it to the MinION
- Check Flowcell (QC it to see how many active pores there are)
- Keep it connected until primed.

Prime the flowcell:

- Remove the bubble from the port
- Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible.
- Set a P1000 pipette to 200 µl
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip
- Load 800 ul of flow cell priming mix
- Wait for 5 minutes
- Open sample port cover
- Load 200 ul of priming mix more into the priming port, observing the bubbles coming out of sample port

Load the library:

- Mix the prepared library gently by pipetting up and down just prior to loading.
- Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
- Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.


HOW TO PRIME A FLOW-CELL ?
1
Flowcell
Sample port
Priming port
Waste port
2
Remove bubble
1000 ul Pipette adjusted to 200 ul
Priming port
200
202
205
210
Insert the tip and start adjusting the volume increasing to 220 slowly
Stop when you see the yellow liquid and take out the tip
3
Priming of port
tip
Priming port
800 ul buffer
First drop just above the port
Then insert the tip inside the port and load the rest
Take the tip out just before the last drop
5 minutes incubation
Open the sample port
Repeat all above on priming port with 200 ul more buffer
You should see bubblings on smaple port as you load the buffer in priming port
4
Sample port
Load the library on sample port in a drop-wise manner from above
Close both ports after loading
Library ready to sequence!
By Oguzhan Begik