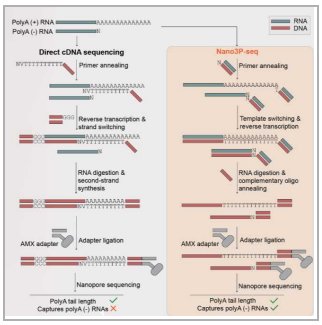

VERSION 2

DEC 20, 2022

WORKS FOR ME 1

Nano3P-seq Protocol V.2

COMMENTS 0

DOI
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ABSTRACT

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.

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PROTOCOL CITATION

Oguzhan Begik 2022. Nano3P-seq Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2 Version created by Oguzhan Begik

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Without Barcoding

1 **Materials and consumables required**

●Direct cDNA Sequencing Kit (ONT, SQK-DCS109)
●Flow Cell Priming Kit (ONT, EXP-FLP001)
●AMPure XP Reagent (Agencourt , A63881)
●Blunt/TA Ligase Master Mix (NEB, M0367)
●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 (Standarc	/5Phos/CTTCCGATCACTTGCCTGTCGCTCTATCTTCN
R_RNA (RNase-Fı	rGrArArGrArArUrArGrArGrCrGrArCrArArGrGrCrArArGrUrGrArUrCrGrGrArArG/3SpC3/
CompA_DNA (Sta	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	

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

A	B	C	D
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 m	4 uL	450 mM NaCl, 5 mM MgCl2, 20 mM Tris-HC
DTT	0.1 M	1 uL	5 mM
Pre-annealed oligos	1 uM	2 uL	0.1 uM
RNA		Up to 10 u	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!

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

- 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 Concen	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 AMX Adapter Ligation

22.5 uL cDNA-complement mix
2.5 uL Adapter Mix (AMX adapter from the SQK-DCS109 kit)
25 uL Blunt/TA Ligase Mix

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

Ampure XP Beads Cleanup

- Add 25 ul resuspended AMPure XP beads (0.5X) to the reaction and mix by flicking
- Incubate 10 minutes at room temperature
- Thaw Wash Buffer (WSB) 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

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

● Remove the supernatant
● Add 200 ul WSB 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 WSBcompletely 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 13 ul Elution Buffer (EB)
● Incubate5-10 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
● 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.

With Barcoding

2 Materials and consumables required

● Direct cDNA Sequencing Kit (ONT, SQK-DCS109)
● Native Barcoding Expansion 1-12 (EXP-NBD104)
● Flow Cell Priming Kit (ONT, EXP-FLP001)
● NEBNext Quick Ligation Module (E6056)
● AMPure XP Reagent (Agencourt , A63881)
● Blunt/TA Ligase Master Mix (NEB, M0367)
● 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 (Standa	/5Phos/CTTCCGATCACTTGCCTGTCGCTCTATCTTCN
R_RNA (RNase F	rGrArArGrArUrArGrArGrCrGrArCrArGrGrCrArArGrUrGrArUrCrGrGrArArG/3SpC3/

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

A	B
CompA_DNA (S	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 Concentratior	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	4 uL	450 mM NaCl, 5 mM MgCl2, 20 mM
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

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

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 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

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 Concentratio	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

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

A	B	C	D
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

2.4 Native Barcode Ligation

22.5 uL cDNA-complement mix
2.5 uL Native Barcode (from the EXP-NBD104 kit)
25 uL Blunt/TA Ligase Mix

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

Cleanup using Ampure XP Beads

●Mix the samples with 25 ul 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 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 16 ul water(Normally its 26 ul but then in the pooling step it makes tomuch volume)
●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
●Pool the barcoded samples at the desired ratio to a final volume of 65 µl in a DNA LoBind 1.5ml Eppendorf tube. Aim for as high a concentration as possible which does not exceed **200 fmoles**total. If the total volume is >65 **µ**l, perform a 2.5x AMPure clean up and elute in 65 **µ**l of nuclease free water.

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

● Taking the pooled and barcoded DNA, perform adapter ligation as follows, mixing by flicking the tube between each sequential addition.

AM II Ligation

65 µl 200 fmol pooled barcoded sample
5 µl Adapter Mix II (AMIIadapter from the EXP-NBD104 kit)
20 µl NEBNext Quick Ligation Reaction Buffer (5X)
10 µl Quick T4 DNA Ligase

●Mix gently by flicking the tube, and spin down.
●Incubate the reaction for 10 minutes at RT.

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 Wash Buffer (WSB) 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 200 ul WSB 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 WSBcompletely 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 13 ul Elution Buffer (EB)
●Incubate5-10 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
●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

Before starting:

●Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB)
●Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.
●Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

Citation: Oguzhan Begik Nano3P-seq Protocol https://dx.doi.org/10.17504/protocols.io.3byl4j292lo5/v2

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

●In the new tube, prepare the following:
·37.5 µl Sequencing Buffer (SQB)
·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:

- Mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing, spin down and return to ice.
- Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.
- 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
- Add 30 ul FLT into a tube of FB
- Mix by pipetting up and down
- Load 800 ul of buffer
- Wait for 5 minutes
- Open sample port cover
- Load 200 ul of buffer 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.

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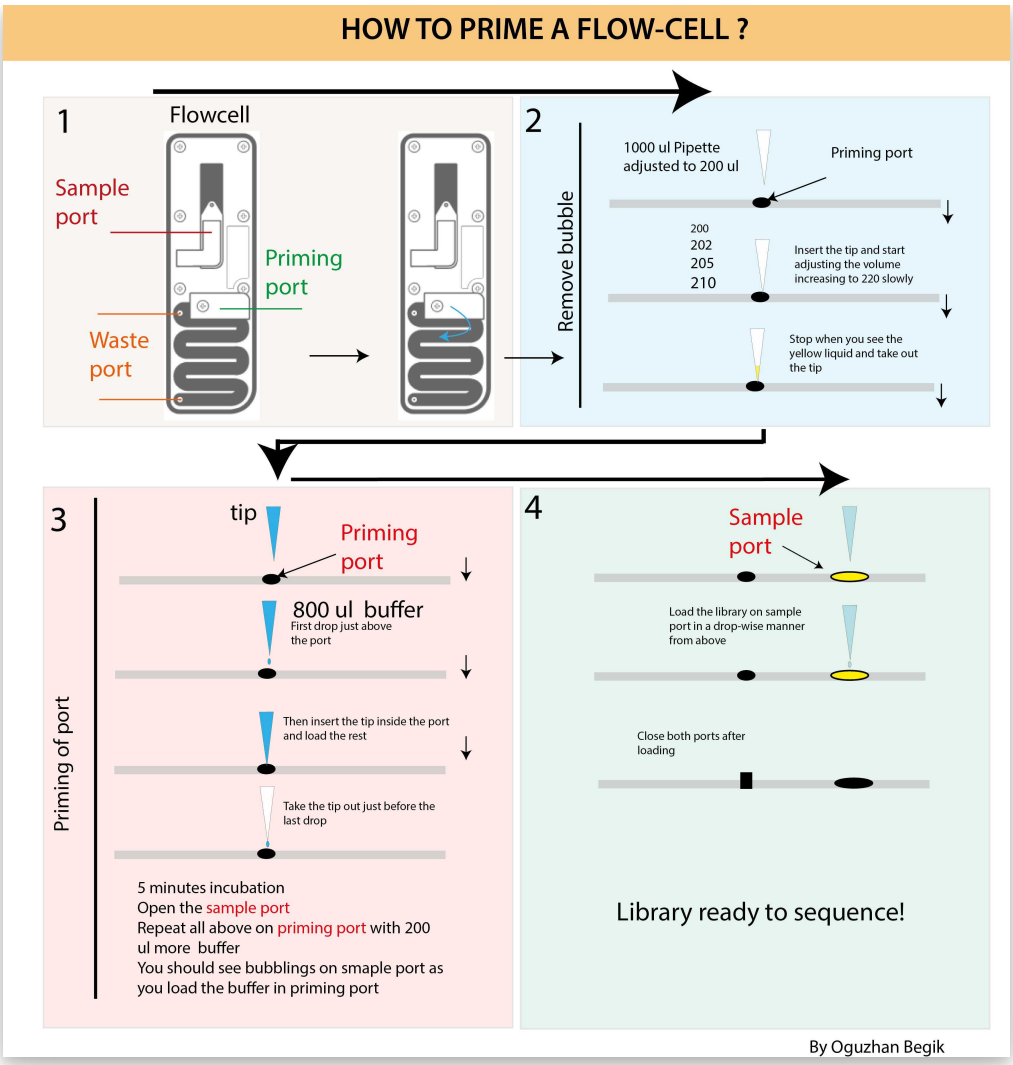

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