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HIV-1 partial-*pol* nanopore sequencing protocol

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We use this protocol and it's working

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Abstract

Reverse transcription and amplification of the HIV-1 partial-*pol* gene were performed using primers and PCR conditions adapted from **Zhou et al. (2011)**, originally optimized for drug resistance surveillance in resource-limited settings. The resulting amplicons (~1.3 kb, covering the protease (PR) and reverse transcriptase (RT) regions) were used for nanopore library preparation.
Optionally, a nested PCR can be performed using the second-round primers described in the same study, yielding a product of 1,084 nt. However, we found that a single round of RT-PCR was sufficient for successful amplification, reducing both time and cost.

Library preparation followed a modified version of the **ARTIC LoCost protocol**, adapted for partial-*pol* amplicons and compatible with the Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies).

Bioinformatics Workflow Suggestion:
RAMPART can be run concurrently with ONT MinKNOW sequencing to provide real-time visualisation of read mapping, coverage, and reference matching to the HXB2 genome for each barcode. The RAMPART configuration protocol is available at https://github.com/AnyaKovalenko/rampart-protocol-HIV-1_partial-pol
For reference-based consensus generation, align reads to the HXB2 reference genome (GenBank accession: **K03455.1**), or specifically to the pol region (coordinates: 2030–3400), using Minimap2, followed by polishing with Racon and Medaka.

Please cite the associated publications when using or adapting this workflow.

Materials

	A	B	C
	Reagent	**Supplier**	**Catalog #**
	QIAamp Viral RNA Mini Kit (50)	Qiagen	52904
	Nuclease free water	Omega Bio-Tek	PD092
	SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase	Invitrogen	12574035
	PCR Clean - DX	Aline BioSciences	C-1003-5
	QuantiFluor ONE dsDNA System	Promega	E4870
	Thermo Scientific GeneRuler 1kb DNA Ladder, ready-to-use	Thermo Scientific	10809360
	All-in-one tablets with pre-measured agarose, TBE, and SeeGreen stain	GELATO	RG-1500-20
	10X TBE Buffer (Tris Borate EDTA) - 500 mL	ufcbio	BTBE-10X
	HIV pol primers	IDT	
	NEBNext Ultra II End Repair/dA-tailing module	New England BioLabs	E7546S
	Blunt/TA Ligase Master Mix	New England BioLabs	M0367S
	NEBNext Quick Ligation Module	New England BioLabs	E6056S
	Native Barcoding Kit 96 V14	ONT	SQK-NBD114.96
	Flow Cell Wash Kit	ONT	EXP-WSH004
	Flow cells R10.4.1	ONT	FLO-MIN114
	Ethanol (96–100%)	Generic	

I. RNA Extraction

1 QIAamp Viral RNA Minikit (Qiagen), following the manufacturer's instruction

II. One-Step RT-PCR

2 NOTE: Prepare primer:

	A	B	C	D	E	F	G
		Primer Name	**Sequence 5'-3'**	**Type**	**Product Size**	**Position in HXB2**	**Purpose**
	1	PRTM-F1a*	TGAARGAITGYACTGARAGRCAGGCTAAT	forward	1,300 nt	2057–2085	One-Step RTPCR
		PRTM-F1b*	ACTGARAGRCAGGCTAATTTTTTAG			2068–2092	
	2	RT-R1	ATCCCTGCATAAATCTGACTTGC	reverse		3370–3348	

*PRTM-F1 is a mixture of primers F1a and F1b at a ratio of 1:1 (w/w) and is used as the forward primer.

Note

*The table includes primers for the One-Step RT-PCR only. If you also wish to perform the nested PCR step, please refer to **Zhou et al. (2011)**. Once amplification is complete, you may proceed with the library preparation steps outlined in this protocol. We found that a single round of RT-PCR was sufficient for successful amplification, reducing both time and cost.*

3 Prepare 100 µM primer stocks for all primers: add NFW or TE to the primer based on its amount (in nanomoles) to achieve the desired concentration.
Mix equal volume of the 100 µM primer stocks of PRTM-F1a and PRTM-F1b in one tube: ei., 40ul + 40ul =
80ul (now this is your mixture of the forward primer 100 µM). Prepare 10 µM working solution for both forward and reverse primers.

4 Prepare RTPCR reaction mix for the required number of reactions plus 1-2 additional reaction for overage. A negative control (without template RNA) should be included in

every experiment:

	A	B
	Component	**Volume (ul) per rxn**
	2X Reaction Mix	25
	SSIII RT/Platinum Taq High Fidelity Enzyme Mix	1
	10 µM forward PRTM-F1ab	1
	10 µM reverse RT-R1	1
	template RNA	20*
	NFW	2
	Total	**50**

*for high viral load samples 10 ul RNA can be used.

5 Start the following program on the PCR thermocycler:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	RT	50°C	45 min	1
	Enzyme inactivation	94°C	2 min	1
	Denature	94°C	15 sec	40
	Anneal	50°C	20 sec	
	Extend	72°C	2 min	
	Final extension	72°C	10 min	1
	Hold	4°C	-	-

Note

PCR product can be confirmed by 1% agarose gel electrophoresis. Based on our experience, the bands may appear faint or barely visible for samples with low viral load, but this is typically sufficient for successful sequencing.

III. Amplicon Clean Up

11m 30s

6 Add 50 µL of magnetic beads (1× ratio) to each well/tube. Mix gently by pipetting (or by flicking). Briefly spin down and incubate for 00:05:00 at Room temperature. 5m

7 Place on magnetic rack and incubate for 00:05:00 or until the beads have pelleted and the supernatant is completely clear. 5m

8 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

9 Keep on magnet, wash the pellet with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. After 00:00:30 remove the ethanol using a pipette and discard being careful not to touch the bead pellet. 30s

10 Repeat the ethanol wash step go to step #9

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

12 With the tube lid open allow the pellet to dry for 00:01:00 or until the pellet loses its shine, but do not dry the pellet to the point of cracking. 1m

13 Remove the tube from the magnetic rack and resuspend pellet in 25 µL NFW, mix gently by flicking and incubate for 00:05:00 at Room temperature.

14 After the incubation place on magnet. Pellet the beads on the magnet until the eluate is clear and colourless (~3min) and elute 25 µL to a clean 1.5 mL Eppendorf LoBind tube ensuring no beads are transferred into this tube.

IV. Quantification

15

Note

use only thin-wall, clear, 0.5 ml PCR tubes (acceptable tubes include Qubit/Quantus assay tubes).
The fluorometer should be calibrated before use. Please follow the calibration instructions specific to your instrument.

Take 1 µL of sample for quantification with Quantus using QuantiFluor ONE dsDNA Dye. Record the final sample concentration results.

Note

PCR product concentrations for the ~1.3 kb HIV-1 pol amplicon may be low (e.g., <1 ng/µL), especially when working with degraded RNA, such as that extracted from dried blood spots (DBS) or low viral load plasma samples. However, successful sequencing with high read depth (e.g., >1000×) is often achievable.

V. End Prep

1h

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

	A	B
	Component	**Volume (ul) per rxn**
	NFW	(optional, if high viral load samples)*
	End Prep Reaction Buffer	1.2
	End Prep Enzyme Mix	0.5
	Amplicons (from previous step)	8.3
	TOTAL	**10 ul**

*Nuclease-Free Water (NFW) is optional and used only to adjust the final reaction volume to 10 µL. If the amplicon concentration is high, you may reduce the amplicon input volume and compensate with NFW (e.g., 3.3 µL of amplicon + 5 µL of NFW). However, if the amplicon concentration is low (e.g., <5 ng/µL), it is recommended to use the maximum possible volume of amplicon (up to 8.3 µL) without NFW to ensure sufficient input for effective library preparation.

17 Mix gently by flicking and briefly spin down.

18 Set-up the following incubation using PCR thermocycler: 1h

20 °C for 00:30:00

65 °C for 00:30:00

4 °C Hold or place on ice

Note

For best results EP products shouldn't be frozen before barcoding. For best results proceed directly to barcode ligation.

VI. Barcode Ligation 40m

19 Select a unique ONT Native barcode (Native Barcoding Kit 96 V14 (SQK-NBD114.96) ONT) for every sample to be run together on the same flow cell.

20 In a new PCR strip-tube set up the following reaction for each sample:

	A	B
	Component	**Volume (ul) per rxn**
	Blunt/TA Ligase Master Mix	5
	Native Barcode (ONT)	1.25
	End-preped amplicons	3.75
	NFW	(optional, if high viral load samples)*
	TOTAL	**10**

*the same idea. See the previous step #16
NFW is optional and used only to adjust the final reaction volume to 10 µL. If the amplicon concentration is high, you may reduce the end-preped amplicon input volume and compensate with NFW (e.g., 1.5 µL of end-preped amplicon + 2.25 µL of NFW).

21 Mix gently by flicking and briefly spin down.

22 Set-up the following incubation using PCR thermocycler: 40m

20 °C for 00:30:00

70 °C for 00:10:00

4 °C Hold or place on ice

Note

You can freeze at -20 °C (eg., overnight) the samples at this point if you need to pause the experiment.

VII. Barcodes Pooling & Clean Up 1 24m

23 POOL barcoded amplicons in a clean 1.5 mL Eppendorf DNA LoBind tube:

- If processing <32 samples, pool all 10 µL from each barcoding reaction.
- If processing 33-47 samples, pool 8 µL from each barcoding reaction.
- If processing 48-80 samples, pool 5 µL from each barcoding reaction.
- If processing 81-96 samples, pool 3 µL from each barcoding reaction.

24 Vortex the SPRI magnetic beads to resuspend. Add 0.4× volume of beads to the pooled reaction and mix by flicking.

25 Incubate the reaction for 00:05:00 at Room temperature . 5m

26 Place the tube into a magnetic rack for 00:05:00 . 5m

27 Keep on magnet and remove all supernatants.

28 Wash the beads with 250 µL SFB (ONT). Remove a tube from the magnetic rack and fully resuspend the pellet in SFB (gently by either flicking or pipetting). Then pulse centrifuge and return the tube to the magnet and allow the beads to pellet. Allow 00:05:00 contact time with the magnet. 5m

29 Repeat the SFB wash go to step #27 and go to step #28

30 Keep on magnet and remove all supernatants. Pulse centrifuge and remove any residual SFB. There is no need to air dry the beads after SFB washes.

31 Keep tube on the magnet and add 200 µL of 80 % Ethanol to bathe the pellet. Carefully remove and discard ethanol, being careful not to touch the bead pellet.

Note

Only perform 1x 80% ethanol wash

32 Pulse centrifuge to collect all liquid at the bottom of the tube and return the tube on a magnet. Carefully remove as much residual ethanol as possible using a P10 pipette.

33 With the tube lid open incubate for 00:01:00 or until the pellet loses its shine (do not allow the pellet dries completely, it will crack and become difficult to resuspend). 1m

34 Remove the tube from the magnetic rack and add 32 µL NFW water. Mix gently by flicking and incubate for 00:05:00 at Room temperature. 5m

35 Place the tube into a magnetic rack for 00:03:00 or until the eluate is clear and colourless. 3m

36 Elute 32 µL sample to a clean 1.5 mL Eppendorf DNA LoBind tube. Be careful not to take any beads! Use light!

37 Take 1 µL for quantification using a fluorometer such as a Qubit or Quatus. Record the metric.

Note

You can store the pooled sample at 4 °C *overnight and continue the library preparation the next day, if needed.*

VIII. Adapter Ligation

20m

38 Set up the following Adapter Ligation reaction:

	A	B
	Component	**Volum (ul)**
	NEB Quick Ligation Buffer	10
	Adapter mix ONT	5
	Quick T4 Ligase	5
	Pooled library	30
	TOTAL	**50**

39 Mix gently by flicking and briefly spin down.

40 Incubate the reaction for 00:20:00 at Room temperature 20m

or if using PCR thermocycler: 20°C for 20 minutes and then 4°C Hold

IX. Final Clean Up

22m

41

Note

1. ***Do NOT use Ethanol!!!*** *for the final clean-up step as it would denature motor proteins on adaptors. SFB will remove excess adapter without damaging the adapter-protein complexes.*
2. *Vortex magnetic beads thoroughly before use to ensure they are well resuspended; the solution should be a homogenous brown colour.*

Add 50 µL magnetic beads (1× ratio), and mix gently by flicking the tube. Pulse centrifuge to collect all liquid at the bottom of the tube.

42 Incubate for 00:05:00 at Room temperature . 5m

43 Place the tube into a magnetic rack for 00:05:00 5m

44 Remove all supernatants and wash beads with 250 µL SFB (ONT). Resuspend beads in SFB completely by gently pipette mixing/flicking. Pulse centrifuge to collect all liquid at the bottom of the tube.

45 Return on the magnet and allow the beads to pellet. Allow 00:05:00 contact time with the magnet. 5m

46 Repeat the SFB wash step go to step #44 and go to step #45

47 After second SFB wash remove all supernatant. Then spin down and carefully remove any residual SFB. You do not need to allow to air dry with SFB washes.

48 Remove the tube from the magnet. Add 15 µL of EB (ONT) and gently resuspend by flicking. Incubate 00:05:00 at Room temperature . 5m

49 Place the tube into the magnetic rack for 00:02:00 or until the eluate is clear and colourless. 2m

50 Transfer 15 µL of final library to a new 1.5mL Eppendorf DNA LoBind tube. Be careful not to take any beads!

51 Take 1 µL for quantification using a fluorometer such as a Qubit or Quatus. Record the metric.

X. MinION Sequencing

52 Prime and load the flow cell with the final library, following the current ONT protocol for the Native Barcoding Kit you are using (Native Barcoding Kit 96 V14).

Note

*For detailed instructions on priming and loading a MinION flow cell, see this **video***

53 Start the sequencing run using MinKNOW.

Note

If using live basecalling, make sure to enable "Barcode both ends" in the basecalling settings.
We also recommend using High Accuracy basecalling mode and enabling the "Trim barcodes" option in the barcoding settings.

HAPPY SEQUENCING!!!