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Amplification and sequencing of HTLV-1 whole-genome using Nanopore

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Virology IMT

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Abstract

Human T-lymphotropic virus type 1 (HTLV-1) remains frequently underrecognized despite its association with severe diseases in 3–5% of infected individuals. This lack of awareness, coupled with the limited availability of complete HTLV-1 genome sequences in public databases, impairs progress in understanding the virus. Current sequencing techniques, such as Sanger and Illumina, are often costly, and/or time-consuming. Expanding our knowledge of HTLV-1's genomic structure is crucial for advancing our understanding of its basic biology, improving epidemiological studies, and investigating how genetic variation contributes to the diverse clinical manifestations of HTLV-1-associated diseases.
To address these gaps, we developed an efficient and cost-effective method for amplifying and sequencing the complete HTLV-1 genome using Nanopore technology on the MinION platform. This approach offers significant advantages, including real-time sequencing, portability, and relatively low cost compared to traditional methods. Our protocol can facilitate studies on HTLV-1's genetic diversity and pathogeny.

Materials

- **Primer list**

	A	B	C	D
	Name	**Pool**	**Sequence**	**Size**
	HTLV-1_1_Left	1	ATAAGCTCAGACCTCCGGGAAG	22
	HTLV-1_1_Right	1	GAAAACGAAACAAAGACGCAGAGT	24
	HTLV-1_2_Left	2	AAAAGCGTGGAGACAGTTCAGG	22
	HTLV-1_2_Right	2	GAGTGCTATAGAATGGGCTGTCG	23
	HTLV-1_3_Left	1	ACCCCTTTCCCTTTCATTCACG	22
	HTLV-1_3_Right	1	CTTTTGGGAGTAGGCTGGCT	20
	HTLV-1_4_Left	2	CGGTCCCTCCAGTTACGATTTC	22
	HTLV-1_4_Right	2	CAAGCCGGATGGTCTGCATAAA	22
	HTLV-1_5_Left	1	CTTCCAGTCATGCACCCACAT	21
	HTLV-1_5_Right	1	CAGGAAGGGTCTTTGGCACT	20
	HTLV-1_6_Left	2	GTTATAACCCCTTAGCCGGTCC	22
	HTLV-1_6_Right	2	AGGCTGGACAACTAACACTTTGG	23
	HTLV-1_7_Left	1	GGACACACTAATAGCCCTCTAGGA	24
	HTLV-1_7_Right	1	CGGTATAACTGGCAGAATAGATGCT	25
	HTLV-1_8_Left	2	GCTGACATCCCACACCCAAAA	21
	HTLV-1_8_Right	2	ACGACCTATGATGGCCCAGTT	21
	HTLV-1_9_Left	1	CTGTGCTAATACGCCTCCCTTT	22
	HTLV-1_9_Right	1	GGGAAGATGATGAGAGATCTATGGTTAG	28
	HTLV-1_10_Left	2	TCCCAGTTAAAAAGGCCAATGGA	23
	HTLV-1_10_Right	2	CTTGCCAGGAGAATGTCATCCA	22
	HTLV-1_11_Left	1	AAATGCAGCTGGCCCATATCC	21
	HTLV-1_11_Right	1	TCTCGGGGATCAGTATGCCTTT	22
	HTLV-1_12_Left	2	TTGGCGAGATTCAGTGGGTCT	21
	HTLV-1_12_Right	2	TGAAGGTTTGGGTGGAGATGTT	22

	A	B	C	D
	HTLV-1_13_Left	1	GTGTTCCAGTCCAAGCAGCA	20
	HTLV-1_13_Right	1	GGGAGGTAGATCCGTCTGAAAAC	23
	HTLV-1_14_Left	2	GCTCCTGTGAAAGCCCTCAT	20
	HTLV-1_14_Right	2	TAGATTGGTATGGCTGCGAACG	22
	HTLV-1_15_Left	1	ATCATTACCTTCGGACCCTTGC	22
	HTLV-1_15_Right	1	GAAATGGGTAATGTCGCCTTGC	22
	HTLV-1_16_Left	2	CGCCTGCCGCAAAAATAACC	20
	HTLV-1_16_Right	2	GGGTTTTAAGAATGCCATTAGAGCG	25
	HTLV-1_17_Left	1	AAGACTTCCTCAATATGTGTACCTCC	26
	HTLV-1_17_Right	1	AGAACTAGCGCTTACCGGGAT	21
	HTLV-1_18_Left	2	TTAATAGCCGCCAGTGGAAAGG	22
	HTLV-1_18_Right	2	AGCTAGAGTAACCTACTAGATTAGGGC	27
	HTLV-1_19_Left	1	AGCCAGTTTGTTCGTGGACC	20
	HTLV-1_19_Right	1	CGGTATTGAGGAACCAGATGGG	22
	HTLV-1_20_Left	2	TCTCCATTTTTCGAAATGCGGTTT	24
	HTLV-1_20_Right	2	CTTGAATCTGGGGGTCAAAGCA	22
	HTLV-1_21_Left	1	TTTACCCATCGTTAGCGCTTCC	22
	HTLV-1_21_Right	1	AACAGGAGATCAAGGCCTCGT	21
	HTLV-1_22_Left	2	ACTCAAGCAATAGTCAAAAACCACAA	26
	HTLV-1_22_Right	2	GTGCTTGGTTTACAGGGATGACT	23
	HTLV-1_23_Left	1	CATGCATCCTCCGTCAGCTA	20
	HTLV-1_23_Right	1	AAGGAAGGAAGAGGAGAAGGCA	22
	HTLV-1_24_Left	2	CTCCTGCTCTTCCTGCTTTCTC	22
	HTLV-1_24_Right	2	ATCTGTAGGGCTGTTTCGATGC	22
	HTLV-1_25_Left	1	TCCAAGGATAATAGCCCGTCCA	22
	HTLV-1_25_Right	1	TGTATGAGTGATTGGCGGGGTA	22
	HTLV-1_26_Left	2	AGTTCCTTATCCCTCGACTCCC	22

	A	B	C	D
	HTLV-1_26_Right	2	CCTGTGGTAAGGGAGATTTTATAGAGG	27
	HTLV-1_27_Left	1	CTCCTGCCCCACGTGATTTTT	21
	HTLV-1_27_Right	1	AGTACTGTATGAGGCCGTGTGA	22
	HTLV-1_28_Left	2	TCTTTAGTACTACAGTCCTCCTCCTTT	27
	HTLV-1_28_Right	2	ACAAACATGGGGAGGAAATGGG	22
	HTLV-1_29_Left	1	ACACCAACATCCCCATTTCTCTAC	24
	HTLV-1_29_Right	1	CCTGAACTGTCTCCACGCTTTT	22
	HTLV-1_30_Left	2	CGACAACCCCTCACCTCAAAAA	22
	HTLV-1_30_Right	2	GCAGTTAGTCGTGAATGAAAGGGA	24

- **Reagents list:**

	A	B	C
	Component	**Supplier**	**Catalog number**
	QIAamp DNA Mini Kit	QIAGEN	51306
	Q5 Hot Start High-Fidelity 2x Master Mix	NEB	M0494
	NEBNext Ultra II End Repair/dA-tailing module	NEB	E7546
	Native Barcode Expansion Kit 96 V14	ONT	EXP-NBD196
	Blunt/TA Ligase Master Mix	NEB	M0367
	NEBNext Quick Ligation Module	NEB	E6056S
	AMPure XP beads	Beckman	A63881
	Nuclease-free water	NEB	B1500S
	Flow Cell Priming Kit	ONT	EXP-FLP004
	Bovine serum albumin (BSA) 50mg/mL	Sigma-Aldrich	A4737
	Qubit® dsDNA HS Assay Kit	ThermoFisher	Q32854
	70% Ethanol		

- **Consumables:**

	A	B	C
	Component	**Supplier**	**Catalog number**
	DNA LoBind® Tubes 1.5 mL	Eppendorf	0030108051
	Qubit® Assay Tubes	ThermoFisher	Q32856
	MinION Flow cell R10.4.1	ONT	FLO-MIN114
	8-strip PCR tubes or 96-well PCR plates with heat-sealing film	any*	
	Tips with filter (different volumes)	any*	

- **any - check for the best option for your convenience**

Equipaments:

	A	B	C
	Component	**Supplier**	**Catalog number**
	Magnetic rack	ThermoFisher	12321D
	Qubit®	ThermoFisher	Q33238
	Thermocycler		
	HulaMixer® Sample Mixer	ThermoFisher	15920D

Total DNA extraction

30m

1 Recover the total DNA from 200 µL of the biological Sample . The best sample for HTLV amplification is peripheral blood mononuclear cells (PBMC), which can be obtained through a standardized density gradient technique (Ficoll-Paque). In addition to PBMCs, HTLV can also be detected in other biological samples, such as whole blood, or cerebrospinal fluid (CSF). DNA can be extracted using commercial kits. We used the QIAamp DNA Mini Kit, following the manufacturer's instructions, and eluted the extracted DNA in 50 µL of EB buffer or nuclease-free water (NFW).

Primers

1h

2 Reconstitute the lyophilized primers to [M] 100 micromolar (µM) with NFW or buffer.

1h

2.1 Briefly vortex, spin, and allow them to sit for about 00:05:00 . The primers are then ready for use.

2.2 Prepare the Pools 1 and 2 according to the number of each primer pair in separate 1.5 mL microcentrifuge tubes. **Primers with odd numbers will constitute Pool 1, while primers with even numbers will constitute Pool 2**. Add an equal volume (e.g., 5 µL of a [M] 100 micromolar (µM) solution) of each primer to its respective pool tube.

This ensures that individual reactions overlap between pools, but not within pools.

Pools can be stored at -20 °C .

2.3 Prepare [M] 10 micromolar (µM) primer working solutions for each pool by adding 9 parts of NFW (or buffer) to 1 part of the primer mix. Ensure that the diluent used to resuspend the lyophilized primers is the same used for preparing the [M] 10 micromolar (µM) solutions.

Amplification

4h

3 Thaw the Pools at room temperature, briefly vortex and spin. Also, thaw the Q5 Hot Start Master Mix and homogenize by inversion or pipetting up and down.

3.1 Prepare the master mix separately for each pool as the following:

	A	B
	Reagent	**Volume (µL)**
	Q5 Hot Start High-Fidelity 2x Master Mix	12,5
	Pool 1 or Pool 2 primer mix (10µM)	2
	NFW	8
	Total volume	22.5

Distribute 22.5 µL of each master mix in PCR 8-strip tubes, or PCR plates. Then add 2.5 µL of the same sample DNA for each Pool. Do not forget to include negative controls (NC). The final volume per reaction is 25 µL .

3.2 Vortex and spin the tubes or plates. **If using PCR plates, be sure to seal the plates with aluminum foil sealers.** Then, run the following program on the thermal cycler:

	A	B	C	D
	Step	**Temperature (ºC)**	**Duration**	**Cycles**
	Heat Activation	98	30s	1
	Denaturation	98	16s	20
	Annealing	63	5min	
	Touchdown (-0,1ºC/cycle)			
	Denaturation	98	16s	15
	Annealing	61	5min	
	Hold	4	∞	

DNA quantification and dilution (post amplification) 1h

4 DNA quantification can be performed using a fluorometric quantification method, such as the Qubit Fluorometer with theQubit dsDNA HS Assay Kit (Thermo Fisher). Note that samples with concentrations greater than 30 ng/µL should be diluted. In this step, the products from Pool 1 and Pool 2 are finally combined.

Vortex and spin the tubes or plates before diluting the samples.

	A	B	C	D
	Concentrations >30 ng/uL		***Concentrations <30 ng/uL***	
	Component	**Volume (uL)**	**Component**	**Volume (uL)**
	PCR Product Pool 1	2.5	PCR Product Pool 1	24
	PCR Product Pool 2	2.5	PCR Product Pool 2	24
	NFW	45	NFW	2
	Total volume	**50**	**Total volume**	**50**

A 96-well PCR plate can be used to perform dilutions.

End-repair and dA-tailing

30m

5 Before starting, thaw the next reagents at room temperature and **ensure the NEBNext Ultra II End Prep Reaction Buffer is thoroughly mixed by vortexing. Check for any visible precipitate, and if present, vortex for at least 30 seconds to fully dissolve it. Do not vortex the Ultra II End Prep Enzyme Mix.**

Prepare the following master mix:

	A	B
	Component	**Volume (uL)**
	Ultra II End Prep Reaction Buffer	1.2
	Ultra II End Prep Enzyme Mix	0.5
	NFW	5
	Total volume	**6.7**

5.1 In a new 8-strip/PCR plate, add 6.7 µL of master mix, and 3.3 µL of the previous PCR products combined (diluted or not).

5.2 Seal the plate with a new sealant, homogenize by inversion and briefly spin.

5.3 Incubate at room temperature (~ 20 °C) for 00:15:00 followed by 65 °C °C for 00:15:00 .

5.4 Incubate On ice while preparing the mix for the next step.

Barcoding

30m

6 Remove the barcodes from the freezer, vortex to mix, and briefly spin them down. Place on ice.

6.1 Create a plate map indicating which sample will receive which barcode. Properly identify the plate with Library_name+date+barcoding.

6.2 Prepare each well of the plate as follows:

	A	B
	Component	**Volume (uL)**
	dA-tailed amplicons	1
	Native barcode NB01-NB96	1.25
	Blunt/TA Ligase Master Mix	5
	NFW	2.75
	Total volume	**10**

Seal the plate with new sealant.

6.3 Incubate at room temperature (~ 20 °C) for 00:20:00 , followed by 65 °C for 00:10:00 . Then, incubate On ice for 00:01:00 , or until the next step.

20m

SPRI Clean-up

45m

7 Vortex AMPure beads and prepare a working aliquot. Vortex the aliquot again immediately before use. Combine the previous reactions into a single LoBind tube, and add 0.4x AMPure beads. For example:

	A	B	C	D
	Nº of samples	**Volume per reaction (uL)**	**Volume of beads (uL)**	**Total volume**
	12-24	10	48-96	168-336
	48	5	96	336
	96	2.5	96	336

For a different number of samples, multiply the total sample volume by 0.4 to get the necessary volume of beads.

7.1 Mix by inversion for 00:05:00 to allow the library to bind to the beads.

7.2 Briefly spin and place the tube on the magnetic rack, with the lid opening facing forward, opposite the magnetic side of the rack. When the beads have sedimented on the rack wall and the liquid is completely clear, remove the supernatant with a 100 or 200 µl pipette from the side opposite the pellet. Discard the supernatant. **It is important not to touch the pellet and not to discard beads at this point.**

7.3 Add 200 µL of SFB buffer (Nanopore) and completely resuspend the beads. Spin briefly and place the tube back on the magnetic rack. Wait for the pellet to form again, remove the supernatant and discard.

7.4 Repeat step 7.3.

7.5 Wash the pellet with 200 µL - 400 µL of [M] 70 % (v/v) ethanol. Do not disturb the pellet. Carefully pipette on the wall opposite the beads. Discard the supernatant, close the tubes, briefly spin, and place it back on the magnetic rack.

7.6 Remove the residual ethanol and leave the tube open to air dry until the pellet no longer appears shiny.
CAUTION: do not let the pellet become too dry and crack.

7.7 Elute the pellet in 31 µL of NFW and resuspend the beads, ensuring that all particles have been eluted from the wall of the tube.

7.8 Briefly spin and incubate at 37 °C for 00:10:00. Briefly homogenize every 2 minutes.

7.9 Place the tube on the magnetic rack and wait for the pellet to form. Transfer the eluted liquid to a new LoBind tube.
CAUTION: do not transfer beads to the new tube.

7.10 Quantify 1 µL of the library in the Qubit. **Confirm that the DNA concentration is at least 1.0–2.0 ng/µL to proceed with adaptor ligation**. This accounts for potential loss during the adaptor ligation and subsequent clean-up steps. If the concentration is lower than this range, consider adjusting the input DNA amount in previous steps, pooling samples, or repeating the barcoding process if sufficient material is available.

Adaptor ligation and clean-up

1h

8 **Before starting the next step, remove the flow cell from the refrigerator and let it rest at room temperature.**

Prepare the following reaction:

	A	B
	Component	**Volume (uL)**
	Product of the previous step	30
	Native Adapter (NA)	5
	NEBNext Quick Ligation Reaction Buffer (5X)	10
	Quick T4 DNA Ligase	5
	Total volume	**50**

Homogenize by inversion, spin briefly, and incubate the tube at Room temperature room temperature for 00:20:00

8.1 Briefly spin the tube and add 1:1 AMPure beads (50+50). Homogenize by inversion for 00:05:00

8.2 Briefly spin and incubate at Room temperature for 00:10:00

8.3 Place the tube on the magnetic rack, with the lid opening facing forward. Once the beads have sedimented against the wall of the tube and the supernatant is completely clear, carefully aspirate the supernatant using a 100 or 200 µL pipette from the side opposite the pellet. Discard the supernatant without disturbing the pellet or the beads.

8.4 Add 200 µL of SFB buffer and completely resuspend the beads. Spin briefly and place the tube back on the magnetic rack.

8.5 Wait for the pellet to form again, remove the supernatant and discard.

8.6 Repeat steps 8.4 and 8.5. Remove the residual SFB.
ATTENTION: in this step, there is no need to wash the pellet with ethanol.

8.7 Resuspend the pellet in 13 µL of EB buffer (Elution Buffer) and resuspend the beads, making sure that all particles have been eluted from the tube wall.

8.8 Briefly spin and incubate at 37 °C for 00:10:00 Briefly homogenize every 2 minutes.

8.9 Place the tube on the magnetic rack and wait for the pellet to form. Transfer the eluted liquid to a new LoBind tube. **Make sure that no beads are transferred with the eluted.**

8.10 Quantify 1 µL of the library in the Qubit. Then, dilute the library 10-20 fmol in 12 µL of EB.

ATTENTION: with the R.10.4.1 flow cell, only 10-20 fmol of DNA are needed for sequencing. Loading more than 20 fmol of DNA may reduce the duplex read capture rate.

For 400 bp amplicons, 20 fmol = 4.928 ng.
Example = 12.1 ng/uL
1 µl - 12.1 ng
x µl - 4.928 ng
x = 0.41µL (from library) + 11.59 µL EB

Values can be checked here: https://nebiocalculator.neb.com/#!/dsdnaamt

Preparing the Flow cell (loading) 40m

9 **Before using the flow cell, it is a good idea to perform a new pore check (QC) in MinKnow. Once the check is complete, take the flow cell positioned in the MinION to the location where the library will be added.**

9.1 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT), and a tube of Flow Cell Flush (FCF) at room temperature before mixing with a vortex. Then, briefly spin and incubate on ice.

9.2 Prepare the following mix:

	A	B
	Component	**Volume (uL)**
	Flow Cell Flush (FCF)	1,170
	Bovine serum albumin (BSA) 50mg/mL	5
	Flow Cell Tether (FCT)	30
	Total Volume	1,205

9.3 Turn the inlet port cap 90° clockwise so that the opening (Priming port) is visible.

9.4 Take a P1000 pipette and adjust the volume to 800 µL with a tip. Place the tip in the inlet port and, holding it perpendicular to the plane of the flow cell, remove any air by turning the volume knob counterclockwise.

9.5 Load 800 µL of the mix (FCF + FCT + BSA) through the Priming port, dispensing slowly and gently to avoid introducing air bubbles. Wait for 5 minutes.

9.6 In the library tube, prepare the following mix, with a total volume of 75 µL for sequencing:

	A	B
	Component	**Volume (uL)**
	Sequencing Buffer (SB)	37.5
	Library Beads (LIB) or Library Solution (LIS)*	25.5
	Standardized library	12
	Total volume	75

Vortex the LIB tube immediately before use.

9.7 Carefully lift the lid of the SpotON port and pipette an additional 200 µL of the mix (FCF + FCT + BSA). This will initiate a siphon in the SpotON port (it works by creating a continuous flow of liquid) to allow the library to be loaded.

9.8 Add the 75 µL of the library through the SpotON, dripping continuously. Make sure each drop enters the siphon port before adding the next;

9.9 Gently close the SpotON lid, making sure the cap goes into the SpotON port. Close the Priming port and close the MinION lid.

MinION Sequencing

10m

10 **Before starting the run, check the internet connection and available computer memory. Depending on the kits you used to prepare your sample, some parameters described below may differ.**

10.1 Connect the MinION to the computer and wait for the MinION and flow cell to be recognized by the software.

10.2 Select the flow cell and enter the name of the experiment. For this protocol standardization, the flow cell type used was FLO-MIN114.

10.3 Select the kit used, which may be SQK-NBD 114.96 (96 barcodes) or SQK-NBD 114.24 (24 barcodes).

10.4 Adjust the run settings: time, minimum, and maximum read length.

10.5 Select Basecalling ON and choose the type of basecalling. It is recommended to use at least the High-accuracy. However, be aware that it requires greater computational capacity and GPU to run in real-time without issues.

10.6 It is also possible to trim the barcodes. To do this, select Trim barcodes and Barcodes both ends.

10.7 Select where you want to save the generated sequencing data.

10.8 Select Filtering ON.

10.9 START RUN

Bioinformatics pipeline analysis

45m

11 The bioinformatics pipeline used is available at: https://github.com/filiperomero2/ViralUnity, which is compatible with Nanopore long reads.

Bed file: htlv-1.primer.bed 3.8KB
Reference sequence used: NCBI Reference Sequence NC_001436.1

Protocol references

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F., Beutler, N. A., Burton, D. R., Lewis-Ximenez, L. L., de Jesus, J. G., Giovanetti, M., Hill, S. C., Black, A., Bedford, T., Carroll, M. W., Nunes, M., Alcantara, L. C., Jr, ... Loman, N. J. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature protocols*, *12*(6), 1261–1276. https://doi.org/10.1038/nprot.2017.066