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HIV WGS - 400bp Amplicon Tiling - Oxford Nanopore Technology Protocol

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Public Health

Eugene Yeboah
Association of Public health Laboratories, Oregon State Publ...

DISCLAIMER

ABSTRACT

OSPHL in collaboration with APHL, will evaluate the performance of the Oxford Nanopore Technology sequencers for HIV genome sequencing using a modified protocol of the ARTIC amplicon v3:

Nanopore Protocol PCR tiling of SARS-CoV-2 virus with rapid barcoding (SQK-RBK110.96) Library preparation Version: PCTR_9125_v110_revE_24Mar2021

The modifications include:

- Switching to the Nanopore RAPID barcoding, which requires less time (10 min) and fewer reagents.
- HIV Primer sequences designed by Primal Scheme.

HIV-1:
https://www.dropbox.com/sh/xnhpektoen7chdd/AAAT789gnEnwbbCjF_EMuRu8a?

Citation: Eugene Yeboah, Noah C Hull HIV WGS - 400bp Amplicon Tiling - Oxford Nanopore Technology Protocol https://dx.doi.org/10.17504/protocols.io.e6nvwj5p2lmk/v1

dl=0
HIV-2:
https://www.dropbox.com/sh/2sco4ffr30ids7a/AAD0z-VXSXElamsuys7zsmpLa?dl=0

: Primer Sequences

HIV-1:

A	B	C	D	E	F
name	pool	seq	size	%gc	tm (use 65)
HIV-1_v1.0_1_LEFT	1	TGGTTAGACCAGATCTGAGCCT	22	50	60.48
HIV-1_v1.0_1_RIGHT	1	TTTCTTTCCCCCTGGCCTTAAC	22	50	60.68
HIV-1_v1.0_3_LEFT	1	GCTTTAGACAAGATAGAGGAAGAGCA	26	42.31	60.85
HIV-1_v1.0_3_RIGHT	1	TTCCTGCTATGTCACTTCCCCT	22	50	61.02
HIV-1_v1.0_5_LEFT	1	TTGGATGACAGAAACCTTGTTGG	23	43.48	59.62
HIV-1_v1.0_5_RIGHT	1	AAGAAAATTCCCTGGCCTTCCC	22	50	61.01
HIV-1_v1.0_7_LEFT	1	ATTAGAAGAAATGAGTTTGCCAGGAA	26	34.62	59.72
HIV-1_v1.0_7_RIGHT	1	TTCTTTATGGCAAATACTGGAGTATTGT	28	32.14	59.98
HIV-1_v1.0_9_LEFT	1	GAGACACCAGGGATTAGATATCAGT	25	44	59.61
HIV-1_v1.0_9_RIGHT	1	CCCTGGGTAAATCTGACTTGCC	22	54.55	61.13
HIV-1_v1.0_11_LEFT	1	AGAGCCATTTAAAAATCTGAAAACAGGA	28	32.14	60.77
HIV-1_v1.0_11_RIGHT	1	CAGTCTTCTGATTTGTTGTGTCAGT	25	40	60.08
HIV-1_v1.0_13_LEFT	1	GTCAGTGCTGGAATCAGGAAAGT	23	47.83	61.06
HIV-1_v1.0_13_RIGHT	1	CGTAGCACCGGTGAAATTGCT	21	52.38	61.64

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HIV-1_v1.0_15_LEFT	1	AGACATAATAGCAACAGACATACAAACT	28	32.14	59.77
HIV-1_v1.0_15_RIGHT	1	CCAATCTAGCATCCCCTAGTGG	22	54.55	59.88
HIV-1_v1.0_17_LEFT	1	CAAGCAGGACATAACAAGGTAGGA	24	45.83	60.65
HIV-1_v1.0_17_RIGHT	1	TCCAGGGCTCTAGTCTAGGATC	22	54.55	59.81
HIV-1_v1.0_19_LEFT	1	TCTCTATCAAAGCAGTAAGTAGTACATGT	29	34.48	60.52
HIV-1_v1.0_19_RIGHT	1	GCATGTGTGGCCCAAACATTAT	22	45.45	60.28
HIV-1_v1.0_21_LEFT	1	AGCGGGAGAATGATAATGGAGAA	23	43.48	59.61
HIV-1_v1.0_21_RIGHT	1	GCATTGTCCGTGAAATTGACAGA	23	43.48	60.31
HIV-1_v1.0_23_LEFT	1	AGCTAGCAAATTAAGAGAACAATTTGGA	28	32.14	60.61
HIV-1_v1.0_23_RIGHT	1	TTCACTTCTCCAATTGTCCCTCA	23	43.48	59.86
HIV-1_v1.0_25_LEFT	1	CTATTGAGGCGCAACAGCATCT	22	50	61.5
HIV-1_v1.0_25_RIGHT	1	ACCTACCAAGCCTCCTACTATCA	23	47.83	60.05
HIV-1_v1.0_27_LEFT	1	ACCACCGCTTGAGAGACTTACT	22	50	61.27
HIV-1_v1.0_27_RIGHT	1	TGCTCCATGTTTTTCCAGGTCT	22	45.45	60.34
HIV-1_v1.0_29_LEFT	1	CACACACAAGGCTACTTCCCTG	22	54.55	61.31
HIV-1_v1.0_29_RIGHT	1	AACCAGAGAGACCCAGTACAGG	22	54.55	61.01
HIV-1_v1.0_2_LEFT	2	TAGAAGGAGAGAGATGGGTGCG	22	54.55	61.19
HIV-1_v1.0_2_RIGHT	2	TTTTGGCTGACCTGATTGCTGT	22	45.45	61.2
HIV-1_v1.0_4_LEFT	2	GCTGCAGAATGGGATAGAGTGC	22	54.55	61.56
HIV-1_v1.0_4_RIGHT	2	TTCTTCTAGTGTAGCCGCTGGT	22	50	61.33
HIV-1_v1.0_6_LEFT	2	GGAAGGACACCAAATGAAAGATTGT	25	40	60.37
HIV-1_v1.0_6_RIGHT	2	TGTCCACAGATTTCTATGAGTATCTGA	27	37.04	59.94

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HIV-1_v1.0_8_LEFT	2	AGTAGAAATTTGTACAGAGATGGAAAAGG	29	34.48	60.47
HIV-1_v1.0_8_RIGHT	2	AAGGCTCTAAGATTTTTGTCATGCT	25	36	59.67
HIV-1_v1.0_10_LEFT	2	CAGCCTATAGTGCTGCCAGAAA	22	50	60.6
HIV-1_v1.0_10_RIGHT	2	TTTGCACTGCCTCTGTTAATTGT	23	39.13	59.62
HIV-1_v1.0_12_LEFT	2	GGGAGACTAAATTAGGAAAAGCAGGA	26	42.31	61.02
HIV-1_v1.0_12_RIGHT	2	AGCCATTGCTCTCCAATTACTGT	23	43.48	60.57
HIV-1_v1.0_14_LEFT	2	GGGCAGGAAACAGCATATTTTCT	23	43.48	59.81
HIV-1_v1.0_14_RIGHT	2	TGCTGTCCCTGTAATAAACCCG	22	50	60.54
HIV-1_v1.0_16_LEFT	2	GGGAAAGCTAGGGGATGGTTTT	22	50	60.48
HIV-1_v1.0_16_RIGHT	2	TCGTAACACTAGGCAAAGGTGG	22	50	60.47
HIV-1_v1.0_18_LEFT	2	GCAACAACTGCTGTTTATCCATTTT	25	36	59.91
HIV-1_v1.0_18_RIGHT	2	TTTCCTATATTCTATGATTACTATGGACCAC	31	32.26	59.61
HIV-1_v1.0_20_LEFT	2	TACCTGTGTGGAAGGAAGCAAC	22	50	60.67
HIV-1_v1.0_20_RIGHT	2	TGCATATTCTTTCTGCACCTTACCT	25	40	60.96
HIV-1_v1.0_22_LEFT	2	GCCAGTAGTATCAACTCAACTGCT	24	45.83	60.94
HIV-1_v1.0_22_RIGHT	2	ACAGTAGAAAAATTCCCCTCCACA	24	41.67	60.22
HIV-1_v1.0_24_LEFT	2	GGGCTGCTATTAACAAGAGATGGT	24	45.83	61.01
HIV-1_v1.0_24_RIGHT	2	AGGTATCTTTCCACAGCCAGGA	22	50	61.02
HIV-1_v1.0_26_LEFT	2	TGGGCAAGTTTGTGGAATTGGT	22	45.45	61.41
HIV-1_v1.0_26_RIGHT	2	ACCAATATTTGAGGGCTTCCCAC	23	47.83	61.14
HIV-1_v1.0_28_LEFT	2	TGGATGGCCTACTGTAAGGGAA	22	50	60.75
HIV-1_v1.0_28_RIGHT	2	AGCTTGTAGCACCATCCAAAGG	22	50	61.06

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HIV-2:

A	B	C	D	E	F
name	pool	seq	size	%gc	tm (use 65)
HIV-2_v1.0_1_LEFT	1	TGCAAGGGATGTTTTACAGTAGGA	24	41.67	60.28
HIV-2_v1.0_1_RIGHT	1	CCAAGTATGGTTGTTCCTGTTATTCA	26	38.46	60.01
HIV-2_v1.0_3_LEFT	1	GCATTGTATTCAGTCGCTCTGC	22	50	60.46
HIV-2_v1.0_3_RIGHT	1	ACTCCGTCGTGGTTTGTTCCT	21	52.38	62.01
HIV-2_v1.0_5_LEFT	1	AAAACATATTGTGTGGGCAGCG	22	45.45	60.53
HIV-2_v1.0_5_RIGHT	1	TCCTCCACTAATTTTACCCATGCAT	25	40	60.73
HIV-2_v1.0_7_LEFT	1	ATTCGCAGCACCCAATACCAG	21	52.38	61.12
HIV-2_v1.0_7_RIGHT	1	GCGGTTAGCATCTCTTCTAGGG	22	54.55	60.47
HIV-2_v1.0_9_LEFT	1	CAGGACACATCATGGCAAACTG	22	50	60.27
HIV-2_v1.0_9_RIGHT	1	TTGTCCCCTAATTCTATTCCTGCT	24	41.67	59.6
HIV-2_v1.0_11_LEFT	1	GGAAAGATGGACCAAGGCTGAA	22	50	60.74
HIV-2_v1.0_11_RIGHT	1	CTTCCATCCTTGTGGCAAGACT	22	50	60.74
HIV-2_v1.0_13_LEFT	1	GGGTTTTCTACTCCAGATGAGAAGT	25	44	60.43
HIV-2_v1.0_13_RIGHT	1	ACTTTTAGGATTTTCTCTTCCTGGTGT	27	37.04	61.14
HIV-2_v1.0_15_LEFT	1	GGTTAACATTTAACCTAGTAGGAGATCCT	29	37.93	60.78
HIV-2_v1.0_15_RIGHT	1	ACTTGTCTGATGCCTTGACTTACT	24	41.67	60.28
HIV-2_v1.0_17_LEFT	1	TCATTATAGTAGCAGTACATGTTGCAA	27	33.33	59.51
HIV-2_v1.0_17_RIGHT	1	CATGTTGATTAGTCTTTCTGCTGGG	25	44	60.54
HIV-2_v1.0_19_LEFT	1	ACTGGATAGTAGTCCCCACCTG	22	54.55	60.55
HIV-2_v1.0_19_RIGHT	1	ATGAGCTTGGGGATAGTTGCAG	22	50	60.61
HIV-2_v1.0_21_LEFT	1	GGGATGTCAGCAAGCTACACAA	22	50	61.06

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HIV-2_v1.0_21_RIGHT	1	GGTTACATCCCGCTCTGAAGTG	22	54.55	61.17
HIV-2_v1.0_23_LEFT	1	TGGTAGGAATCAGCTGTTTGTTGT	24	41.67	61.01
HIV-2_v1.0_23_RIGHT	1	ATGTGGGTGGTCTCGAGGTT	20	55	60.85
HIV-2_v1.0_25_LEFT	1	AGCACTATTGGGATGCTATGAGG	23	47.83	60.25
HIV-2_v1.0_25_RIGHT	1	CTTCCCTCCATCTGCCTCCAAA	22	54.55	62.21
HIV-2_v1.0_27_LEFT	1	AGCAGATAATTAATACCTGGCATAAAGT	28	32.14	59.66
HIV-2_v1.0_27_RIGHT	1	CTGTTGCTGTTGCTGCACTATC	22	50	60.59
HIV-2_v1.0_29_LEFT	1	AATATGACATGGCAGGAGTGGG	22	50	60.34
HIV-2_v1.0_29_RIGHT	1	CAAGGCCACAAGTCGTAACCA	21	52.38	60.91
HIV-2_v1.0_31_LEFT	1	ATTCGCGAGGACTACGAGAGAG	22	54.55	61.55
HIV-2_v1.0_31_RIGHT	1	CCCTTCCTCTTTTTCTAGGTATATGTCT	28	39.29	60.51
HIV-2_v1.0_33_LEFT	1	ACTACAAGGCCTTCACTCTGTAC	23	47.83	60
HIV-2_v1.0_33_RIGHT	1	AGTACCGGCCAAGTACTGGT	20	55	60.56
HIV-2_v1.0_2_LEFT	2	GAGTTTGGGCACAAGTCAGGAT	22	50	61
HIV-2_v1.0_2_RIGHT	2	ACTGGCAGCTTTATTGAAGAGGT	23	43.48	60.5
HIV-2_v1.0_4_LEFT	2	CGAACAGGGACTTGAAGAGGAC	22	54.55	60.79
HIV-2_v1.0_4_RIGHT	2	TGGCAACCTTCTTTTGACTCCA	22	45.45	60.54
HIV-2_v1.0_6_LEFT	2	AAAAGAGGAAACTACCCCGTGC	22	50	60.99
HIV-2_v1.0_6_RIGHT	2	TCTGTAGATGTTCCCTACCGGT	22	50	60.21
HIV-2_v1.0_8_LEFT	2	TGGATGACCCAAACGCTGCTA	21	52.38	62.16
HIV-2_v1.0_8_RIGHT	2	GGGAAGTTGCGAGGCTTCTTTC	22	54.55	62.2
HIV-2_v1.0_10_LEFT	2	GTCACAGCGTACATCGAGGATC	22	54.55	61.03
HIV-2_v1.0_10_RIGHT	2	AGCTGGCCCTCTTTTTCCATTT	22	45.45	60.95

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HIV-2_v1.0_12_LEFT	2	CTGCATTTACCCTACCAGCAGT	22	50	60.54
HIV-2_v1.0_12_RIGHT	2	GCTTTTGGATGTCATTGACTGTCC	24	45.83	60.93
HIV-2_v1.0_14_LEFT	2	ACCAAGAAGAAAAAGAATTAGAGGCA	26	34.62	59.67
HIV-2_v1.0_14_RIGHT	2	TCCTGCTTTTCCCTCTTTTGACT	23	43.48	60.38
HIV-2_v1.0_16_LEFT	2	AGCAGTCTATGTTGCATGGGTC	22	50	60.86
HIV-2_v1.0_16_RIGHT	2	GAAGAGTGCTGTCTGCCTTCCT	22	54.55	62.44
HIV-2_v1.0_18_LEFT	2	ACAATAGAAACAATAGTACTGATGGCAG	28	35.71	60.29
HIV-2_v1.0_18_RIGHT	2	CCTCCTCTAGGTCTTTTGTTCTGT	24	45.83	60.04
HIV-2_v1.0_20_LEFT	2	TCTTGCTTTACGGCAGGTGAAG	22	50	61.31
HIV-2_v1.0_20_RIGHT	2	GCAAGTGCACCCTCTCTTGAAA	22	50	61.51
HIV-2_v1.0_22_LEFT	2	GACATGGAGACACCCTTGAAGG	22	54.55	60.8
HIV-2_v1.0_22_RIGHT	2	CACGCGGGTATGCCATAGAAAA	22	50	61.5
HIV-2_v1.0_24_LEFT	2	TGGCAATGAATTGTAGCAGGGT	22	45.45	60.74
HIV-2_v1.0_24_RIGHT	2	AGCCTGAATAGTTGGTATCATTACATCT	28	35.71	60.77
HIV-2_v1.0_26_LEFT	2	TGTCAGGATTAGTGTTTCACTCTCA	25	40	60.08
HIV-2_v1.0_26_RIGHT	2	GCTGGTTACTGTTGATTCACAGG	23	47.83	60.06
HIV-2_v1.0_28_LEFT	2	CGTGCTAGGGTTCTTGGGTTTT	22	50	61.26
HIV-2_v1.0_28_RIGHT	2	TTCTTGTTGAATTTGGGCTTCTTCT	25	36	60.02
HIV-2_v1.0_30_LEFT	2	CCCGGTTATCTCCAACAGATCC	22	54.55	60.41
HIV-2_v1.0_30_RIGHT	2	CTTGGAACTGCGAGTATTCCCC	22	54.55	61.18
HIV-2_v1.0_32_LEFT	2	TCACATTTTATAAAAGAAAAAGGGGGACT	29	31.03	60.58
HIV-2_v1.0_32_RIGHT	2	CCCTCTTGCTTTCAGTTTTGCC	22	50	60.73

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HIV-2_v1.0_34_LEFT	2	ACTTTCCAGAAGGGGCTGTAAC	22	50	60.41
HIV-2_v1.0_34_RIGHT	2	TGCTAGGGATTTTCCTGCCTTG	22	50	60.81

DOI

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Freed, N. E., Vlková, M., Faisal, M. B., & Silander, O. K. (2020). Rapid and inexpensive whole-genome sequencing of SARS-CoV-2 using 1200 bp tiled amplicons and Oxford Nanopore Rapid Barcoding. Biology Methods and Protocols, 5(1), bpaa014. https://doi.org/10.1093/biomethods/bpaa014CrossRefPubMedGoogle Scholar

KEYWORDS

HIV, Whole Genome Sequecing, WGS, NGS, ONT, Oxford Nanopore Technology

CREATED

Oct 05, 2022

LAST MODIFIED

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PROTOCOL INTEGER ID

70862

MATERIALS TEXT

EQUIPMENT / SUPPLIES / REAGENTS:

A	B
- Diluent (DIL)	
- Elution Buffer (EB)	
- Flush Buffer (FB)	
- Flush Tether (FLT)	
- Loading Beads II (LBII)	
- Loading Solution (LS)	
- Rapid Adapter F (RAP-F)	
- Rapid Barcode Plate (RB96)	
- Sequencing Buffer II (SBII)	
- SPRI beads (SPRI)	
- Storage Buffer (S)	
- Wash Mix (WMX)	
Absolute Ethanol, 200 proof, Molecular Biology Grade	Cat. # T038181000
Biohazard containers for sharps, assorted sizes	
Coverage Spray HB Plus	Steris #1624-77 or VWR #4212-963
Disposable powder-free gloves	
Eppendorf® Centrifuge 5430/5430R	Cat. # EP022620645
Eppendorf™ PCR Cooler	Cat. # 05-403-00

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EQUIPMENT	
Flow Cell Wash Kit	Nanopore Cat# EXP-WSH004
Gentle rotator mixer or HULA mixer	
HIV-1/HIV-2 Primers	IDT – Custom
Kim Wipes	
Low Protein Binding Collection Tubes (1.5 ml)	Cat. # 90410
LunaScript® RT SuperMix Kit	NEB Cat# E3101
Magnetic Separator, suitable for 1.5ml tubes	
MicroAmp™ 8-Cap Strip, clear	Cat. # N8010535
MicroAmp™ Optical 8-Cap Strips	Cat. # 4323032
MicroAmp™ Optical 96-Well Reaction Plate	Cat. # N8010560
myFuge 12 Micro-Centrifuge	Cat. # 50-550-338
ONT Sequencer	
PCR Cabinet – NuAire, model 126-300	Cat. # A35422
Permanent marking pens	
Q5® Hot Start High-Fidelity SX Master Mix	NEB Cat# M0494
Qubit® 4.0 Fluorometer	
Qubit™ Fluorometry 4.0	Cat. # Q33238
Qubit™ 1X dsDNA HS Assay Kit	Cat. # Q33230 or Q33231
Rainin Multichannel Pipettors – 20ml and 200ml	
Rainin Pipettors – 2ml, 20ml, 200ml and 1000ml	
Rainin Repeater Pipettor – 20ml and 200ml	
Rainin 1000ml filter tips, Terra rack	Cat. # 17014967
Rainin 200ml filter tips, Terra rack	Cat. # 17014963
Rainin 20ml filter tips, Terra rack	Cat. # 17014961
Rapid Barcoding Kit:	Nanopore Cat# SQK-RBK110.96

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REAGENTS	
RNase Away™	Fisher Scientific #21-236-21 or equiv.
SimpliAmp™ Thermal Cycler or similar	Cat. # A35422
SpotON Flow Cell (R9.4.1)	Nanopore Cat# FLO-MIN106D
SUPPLIES	
UltraPure™ DNase/RNase-Free Distilled Water	Cat. # 10977015
Vortex Mixer	

DISCLAIMER:

1 **Reverse Transcription**
The LunaScript® RT SuperMix Kit will be used to reverse transcribe HIV RNA for preparation to perform the Primer Pool PCR assay. NOTE: LunaScript® RT SuperMix will be kept in the PCR Clean room.

2 Place 96-well PCR plate on an Eppendorf® PCR cooler (stored at -20oC).

3 Using a repeater pipettor aliquot 4 µl of LunaScript® RT SuperMix into each sample well of a 96-well PCR plate according to plate map.

4 Cover the plate and move the PCR plate containing master mix from the PCR Clean Room to the processing bench for sample addition.

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5 Add 16 µl of RNA extract to the appropriate sample well and gently mix by pipetting up and down. If less than 16 µl is available, make up the volume with nuclease-free water. Total volume = 20 µl per well.

6 Seal the plate with MicroAmp™ 8-cap strips and briefly centrifuge to collect droplets.

7 Return the RT plate to the Eppendorf® PCR cooler until thermocycler reaches 25oC.

8 10. Load the plate into the SimpliAmp™ thermocycler, then run the "HIV REVERSE TRANSCRIPTION" method with the following parameters: Set heated lid to 105oC / Runtime ~14 minutes

REPS	1	1	1	Hold
TIME	2:00	10:00	1:00	∞
TEMP	25oC	55oC	95oC	4oC

9 **PCR Primer Pool Preparation**
Combine the following components to prepare the master mix for each primer pool (A or B). (Allow for pipetting loss by adding ~ 4 reactions per 32 samples.)

10 **PCR Primer Pool Preparation**
Combine the following components to prepare the master mix for each primer pool (A or B). (Allow for pipetting loss by adding ~ 4 reactions per 32 samples.)

NOTE: Use the HIV-1 RT and Primer Pool Preparation Worksheet to calculate the required amount of master mix. This can be made at the same time as the LunaScript® SuperMix RT plate and stored at 4oC until use.

@10µM

Component	Volume
Nuclease-free Water	8.90 µl
HIV-1 Primer Pool (A or B)	1.10 µl
Q5® Hot Start HF 2X Master Mix	12.5 µl

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Total volume per sample 22.5 µl

11 15. Place 96-well PCR plate on an Eppendorf® PCR cooler (stored at -20oC).

12 16. Using a repeater pipettor aliquot 22.5 µl of each master mix into the appropriate wells of the 96-well plate.

13 17. Cover and store at 4oC until reverse transcription protocol is complete.

14 18. Remove the RT plate from the thermocycler and centrifuge.

15 19. Carefully remove the 8-cap strips and using a multi-channel pipettor transfer 2.5 µl of the RT reactions into the appropriate wells of the PCR plate containing the primer pools. Gently mix by pipetting the contents of each well up and down. Total volume = 25 µl per well.

NOTE: It is useful to separate the plate in half with primer pool A in columns 1-6 and primer pool B in columns 7-12.

16 21. Seal the plate with 8-cap strips and briefly centrifuge.

17 22. Load the plate into the SimpliAmp™ thermocycler, then run the “HIV PCR PRIMERS” method with the following parameters: Set heated lid to 105oC / Runtime ~5 Hours

A	B	C	D	E
REPS	1	45X		Hold
TIME	0:30	0:15	5:00	∞
TEMP	98oC	98oC	65oC	4oC

24. Repeat the same primer pool preparation from above (Step 1) and aliquot it into the same

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18 wells as in Step 1 into a new PCR plate in the same wells as the previous plate.

19 25. NOTE: Use the HIV-1 RT and Primer Pool Preparation Worksheet to calculate the required amount of master mix.
@10µM

Component	Volume
Nuclease-free Water	8.90 µl
HIV-1 Primer Pool (A or B)	1.10 µl
Q5® Hot Start HF 2X Master Mix	12.5 µl
Total volume per sample	22.5 µl

20 26. Remove the cDNA plate from the thermocycler and centrifuge.

21 27. Carefully remove the 8-cap strips and using a multi-channel pipettor transfer 2.5 µl of the 1st cDNA products into the corresponding wells of the new PCR plate containing the 2nd set of primer pools.
(2.5 µl of 1st Pool A or B goes into corresponding 2nd Pool A or B of new plate.)

22 29. Gently mix by pipetting the contents of each well up and down. Total volume = 25 µl per well.

23 30. Seal the plate with 8-cap strips and briefly centrifuge.

24 31. Load the plate into the SimpliAmp™ thermocycler, then run the “HIV PCR PRIMERS” method again. Same parameters as above.
Set heated lid to 105oC / Runtime ~5 Hours

A	B	C	D	E
REPS	1	45X		Hold
TIME	0:30	0:15	5:00	∞
TEMP	98oC	98oC	65oC	4oC

25 **Addition of rapid barcodes**

Citation: Eugene Yeboah, Noah C Hull HIV WGS - 400bp Amplicon Tiling - Oxford Nanopore Technology Protocol https://dx.doi.org/10.17504/protocols.io.e6nvwj5p2lmk/v1

Thaw Rapid Barcode Plate and bring SPRI beads to room temperature ~ 1 hour before PCR is complete.

26 35. Spin down the Rapid Barcode Plate.

27 36. Remove the primer-specific cDNA plate from the thermocycler and centrifuge briefly to collect the contents at the bottom of the wells.

28 37. Place the plate on an Eppendorf® PCR cooler.

29 38. Carefully remove the 8-cap strips from the cDNA plate to prevent any splashing.

30 39. Place a clean 96-well PCR plate on a separate Eppendorf® PCR cooler (stored at -20oC) and combine each 25 µl cDNA reaction into a single well in the plate. Mix by pipetting up and down and set aside. Total volume per sample = 50 µl.

31 40. Place another clean PCR 96-well sample plate on a separate Eppendorf® PCR cooler and using a repeater pipettor aliquot 2.5 µl of nuclease-free water into corresponding sample wells. This is the Barcode Attachment Plate (BAP).

32 41. Using a multichannel pipette, transfer 5 µl of the pooled cDNA products to the corresponding well of the Barcode Attachment Plate (BAP) and mix by pipetting.

33 42. Using a multichannel pipette, transfer 2.5 µl of the appropriate Rapid Barcodes to the corresponding well of the Barcode Attachment Plate (BAP) and mix by pipetting. Be careful not to cross-contaminate the different wells. Total volume per sample = 10 µl.

34 43. Seal the plate with 8-cap strips and briefly centrifuge.

35 44. Incubate the plate on the thermocycler using the “RAPID BARCODE ATTACHMENT” protocol with the following parameters:

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REPS	1	1
TIME	2:00	2:00
TEMP	30oC	80oC

36 **Pooling Samples and Clean-up**
Remove the Barcode Attachment Plate (BAP) from the thermocycler and centrifuge briefly to collect the contents at the bottom of the wells.

37 47. Carefully remove the 8-cap strips from the BAP plate to prevent any splashing.

38 48. Pool the barcoded samples into a 5 ml Eppendorf DNA LoBind tube. ~10 µl per sample

39 Pool the barcoded samples into a 5 ml Eppendorf DNA LoBind tube. ~10 µl per sample
For example:

# Samples	24	48	96
Total Volume	~240 µl	~480 µl	~960 µl

40 50. Resuspend the SPRI beads by vortexing.

41 Add an equal volume of resuspended SPRI beads to the pooled samples and mix by flicking the tube.
For example:

# Samples	24	48	96
Volume of SRPI beads	~240 µl	~480 µl	~960 µl

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42 53. Incubate at room temperature on a gentle rotator mixer (~700 rpm) for 5 minutes.

43 54. While incubating, prepare 3 ml of fresh 80% ethanol in nuclease-free water (2400 µl ethanol 600 µl water).

44 55. Spin down the sample tube briefly to bring any liquid down from the cap and place it on the magnet. A pellet will form on the side wall of the tube.

45 56. When the solution clears, keep the tube on the magnet and pipette off the supernatant and discard.

46 57. Without disturbing the beads, add 1.5 ml of the freshly prepared 80% ethanol. Rotate the tube on the magnet to move the beads front to back a few times to wash beads. Remove the ethanol using a pipette and discard.

47 58. Repeat the previous step.

48 59. Briefly spin down the tube and place it back on the magnet. Pipette off any residual ethanol. Allow to dry for 30 seconds. Do not over dry the pellet to the point of cracking.

49 60. Remove the tube from the magnet and resuspend the pellet in 30 µl Elution Buffer (EB). Gently mix by pipetting up and down.

50 61. Incubate for 10 minutes at room temperature.

51 62. Place the tube on the magnet until the eluate is clear and colorless.

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52 63. Remove and transfer the 30 µl eluate (containing the DNA library) into a clean 1.5 ml Eppendorf DNA LoBind tube. Dispose of the pelleted beads. Be careful not to transfer any of the beads.

53 64. Proceed to quantifying the DNA concentration using the Qubit dsDNA HS Assy Kit. (Or any preferred quantification method)

54 **Library quantification and normalization**

Analyze 1 µl of the amplified library using the Qubit™ 4.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit. For more information, see the Qubit™ dsDNA HS Assay Kits User Guide

Determine the amplified library concentration using Qubit™ 4.0 Fluorometer: .

55 68. Prepare the Qubit™ standards as directed in the user guide (10 µl standard + 190 µl Qubit™ dsDNA HS Buffer), mix well, and incubate for at least 2 minutes.

56 69. For each sample, combine 1 µl of the amplified library with 199 µl of Qubit™ dsDNA HS Buffer, mix well, and incubate for at least 2 minutes.

57 70. On the Qubit™ 4.0 Fluorometer home screen, select dsDNA then 1x dsDNA High Sensitivity.

58 71. Measure the prepared standards.

59 72. Select the sample concentration as “ng/µl” with an input value of 1 µl.

60 73. Measure and record the library concentration.

A total volume of 11 µl is required for the following step.

If concentration of the total pool is less than 600ng/µl, use library pool undiluted.

61 76. Add 1 µl of Rapid Adapter F (RAP F) to the 11 µl normalized barcoded DNA and mix gently.

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62 77. Incubate at room temperature for 5 minutes.

63 78. After incubation, place the prepared library in a cold rack until ready to load onto the SpotON flowcell.

64 **Priming and Loading the SpotON Flow Cell**
Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) or Loading Solution (LS, if using), Flush Tether (FLT) and Flush Buffer (FB) at room temperature.

65 81. Mix the SBII, FB and FLT tubes by vortexing. Spin down the SBII and FLT tubes.

66 82. Open the ONT sequencer lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

67 83. QC the SpotON flowcell using the MinKNOW software before proceeding. QC passes if total active pores are >800.

68 84. After QC is complete, move the unit including the flowcell to the bench for priming and loading.

69 85. Slide the priming port cover clockwise to open the priming port.

70 **Prime and load the SpotON Flow Cell**
In a 1.5 ml Eppendorf LoBind tube, prepare the flow cell priming mix by adding 30 µl of Flush Tether (FLT) to 1.17 ml of Flush Buffer (FB) and vortex to mix.

71 88. Open the priming port and check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl)

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72 89. Set a P1000 to 200 µl

73 90. Insert the tip into the priming port

74 91. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.

75 92. Visually inspect that there is continuous buffer from the priming port across the sensor array.

76 93. Load 800 ul of the priming mix into the flow cell via the priming port without introducing bubbles. Wait 5 minutes.

77 **Prepare the library for loading**

Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting up and down. The LBII tube contains a suspension of beads that settle very quickly. It is vital that they are mixed immediately before use.

In a new tube, prepare the library for loading as follows:

Reagent	Volume
Sequencing Buff II (SBII)	37.5 µl
Loading Beads II	22.5 µl
DNA Library	12 µl
Total volume	**75 µl**

78 **Complete the flow cell priming**

Gently lift the SpotON sample port cover to make the sample port accessible.

79 Slowly load 200 ul of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the instruction of bubbles.

80 Immediately, mix the prepared library by pipetting up and down gently.

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81 Add 75 µl of sample to the flow cell via the SpotON port in a dropwise manner. Ensure each drop flows into the port before adding the next.

82 Replace the SpotON sample port cover, making sure it is seated correctly, close the priming port, and close the sequencer lid. Proceed to sequencing

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