Dec 07, 2023 Version 2

An NGS amplicon tiling protocol for HIV-1 drug resistance detection using Illumina® COVIDSeq™ Assay Kit V.2

DOI
dx.doi.org/10.17504/protocols.io.n92ldmq4ol5b/v2

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Protocol Citation: Noah C. Hull, Eugene Yeboah, laura.j.tsaknaridis, Vanda Makris 2023. An NGS amplicon tiling protocol for HIV-1 drug resistance detection using Illumina® COVIDSeq™ Assay Kit. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.n92ldmq4ol5b/v2**Version created by **NGS-Globalhealth APHL**

Protocol status: Working
We use this protocol and it's working

Created: December 07, 2023

Last Modified: December 07, 2023

Protocol Integer ID: 91941

Keywords: hiv genome, hiv drug resistance mutation, immunodeficiency virus drug resistance, hiv drug resistance testing, hiv complete genome, hivseq, performance of the illumina covidseqtm assay, use of the illumina covidseqtm assay, illumina covidseqtm assay, viral replication of hiv, human immunodeficiency virus, hiv-pc-1000-1, hiv-pc-100-1, hiv-pc-100000-1, stanford hiv drug, hiv-pc-1000000-1, such as the illumina covidseqtm assay, hiv-pc-1000-2, hiv-pc-1000-3, hiv-pc-10000-3, hiv-pc-10000-2, hiv-pc-100-2, hiv-pc-100-3, hiv, hiv-pc-1000000-3, hiv-pc-100000-3, hiv-pc-100000-2, hiv-pc-1000000-2, hiv-pc-10-1, recommendations of the international antiviral society, hiv-pc-10-3, hiv-pc-10-2, covidseqtm assay, illumina covidseq, accurate information for drug resistance detection, international antiviral society, drug resistance mutation, library preparation of the diluted viral nucleic extract, samples at the different viral load, illumina covidseq ruo kits reference guide, covidseq assay, viral copy number dilution, drug

Abstract

Summary

Human immunodeficiency virus (HIV) is the pathogen responsible for acquired immunodeficiency syndrome (AIDS) and continues to be a significant global public health issue. HIV can be managed with antiretroviral (ART) drug treatments by suppressing the viral replication of HIV within the infected individual. Unfortunately, due to the nature of the virus, drug resistance can occur, making the ART drug no longer effective. An amplicon-based assay, such as the Illumina COVIDSeqTM Assay (RUO), was adopted to sequence the entire genome of the HIV-1 virus. We modified the
Illumina COVIDSeq™ Assay (RUO) protocol to prepare HIV-1 libraries and sequenced on the Illumina MiSeq. The sequencing data was analyzed by Terra.bio and Exatype NGS. This study demonstrated the utility of the assay to efficiently
sequence near-complete genome of HIV-1 virus up to 488 viral copies and provide accurate information for drug resistance detection.

Background

According to the World Health Organization, an estimated 39.0 million [33.1–45.7 million] people live with HIV at the end of 2022 [1]. UNAIDS reported that 40.4 million [32.9 million–51.3 million] people have died from AIDS-related illnesses since the start of the epidemic [2]. HIV drug resistance testing is crucial for managing and preventing ARV failures for persons undergoing treatment or drug-naive individuals [3]. Traditionally, HIV drug resistance testing is performed by Sanger sequencing of specific regions within the HIV genome to identify drug resistance mutations. Next-generation sequencing (NGS) technologies have recently been implemented to improve sensitivity and reproducibility and reduce the cost per sample by multiplexing.

In this study, the Oregon State Public Health Laboratory (OSPHL), in collaboration with the Association of Public Health Laboratory (APHL), evaluated the performance of the Illumina COVIDSeq™ Assay (RUO) for HIV complete genome sequencing (HIVSeq). We describe a step-by-step HIV-1 virus genome sequencing protocol that leverages the Illumina COVIDSeq™ Assay (RUO), with reagents remaining the same. The modified protocol utilizes HIV-1 primers designed through primal scheme. We demonstrate that whole genome sequencing of the HIV-1 virus can be achieved with extensive sequencing coverage for viral copies up to 488. Thereby expanding the use of the Illumina COVIDSeq™ Assay (RUO)
beyond SARS-CoV-2.

Results

To evaluate the performance of the assay, HIV-1 strain: IIIB positive culture control from ZeptoMetrix (Part #v0801032CF) was extracted and serially diluted to the following concentration: 10^(-1), 10^(-2), 10^(-3), 10^(-4), 10^(-5), and 10^(-6). Library preparation of the diluted viral nucleic extracts was sequenced on the Illumina MiSeq 2 × 150 bp. Viral copies ranged from 48.8 to 4,880,000 viral RNA copies/ul. We evaluated the assay's

performance for accuracy, precision,
sensitivity, specificity, and limit of detection.

HIV-1 primers
designed with Primal Scheme: Complete Primers under MATERIALS section.

To verify the coverage and detect drug-resistant mutations of all samples, we used FASTQ files exported from the MiSeq instrument. We uploaded them to the Terra platform, used the Illumina PE TheiaCov workflow developed by Theiagen, and mapped them to reference NC-001802.1 to generate run and quality metrics. FASTA files were analyzed for drug resistance mutations using Exatype NGS by Hyraxbio. All samples were run in triplicates. Quality metrics and coverage analysis are shown in Tables 1A and 1B. The average percent reference coverage for viral loads of 4,880,000, 488,000,
48,800, 4,880, and 488 were >90%. Reference coverage for negative controls was undetected due to assembly failure. These results showed that the assay performed well in sequencing HIV-1-positive virus.

To evaluate the accuracy of the assay, we leveraged the HIV-1 positive control's mutations. The positive control has a non-polymorphic mutation F227L found in the non-nucleoside reverse transcription. We calculated the percent accuracy based on the ability of the assay to identify this mutation in all 17 samples that passed quality control metrics. Table 2 shows a
100% consistent detection of the F227L mutation in all samples.

We evaluated the analytical sensitivity of the assay based on the HIV-1 positive control drug-resistant profile within the NRTI – Nucleoside Reverse Transcriptase Inhibitors (susceptible), NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitors (3 intermediate and F227L mutation), PI - Protease Inhibitors (susceptible), and INSTI - Integrase Inhibitors regions (susceptible). The assay detected concordant results in 64 out of 68 drug classes and their resistance calls in all the dilution series (64/68) x 100 = 94%). Data is shown in table 4. Within-run precision was calculated based on three replicates from each dilution series (4.880,000, 488,000, 48,000, 4,800, 480, and 48.8 copies) of the HIV-1 subtype B positive control and three replicates of a negative template control. There were expected 17 positive and eight negative
(3 negative and five non-HIV specimens). The miscalculation rate was 0%. Also, Positive Predictive Value =100% and Negative Predictive Value = 100%. Figure 1 demonstrates that sequencing the HIV-1 genome using the COVIDSeq™ Assay (RUO) (on the Illumina MiSeq 2X150) produces >80% coverage of the complete HIV-1 genome with viral copy down to 488 copies/ul.

Analytical specificity and coinfection for HIV-1 genome were evaluated using confirmed positive samples of HAV-Hepatitis A, HBV-Hepatitis B, HCV-Hepatitis C, and two TP-Syphilis. All samples were confirmed to be negative for HIV-1. QC metrics failed for all five samples due to genome assembly failure and no reads, as depicted in Table 6. Another evaluation of analytical specificity of the mutation detected was calculated based on the assay's ability to detect the
mutation, F227C, a rare nonpolymorphic mutation at position 106, at NNRTI - Non-Nucleoside Reverse Transcriptase

Inhibitors region in each of the 17 samples at the different viral loads of (4.880,000, 488,000, 48,000, 4,800, 480, and 48.8). Table 5 shows (17/17) x 100 =100.00% analytical specificity to detect the mutation.

We evaluated the Limit of Detection by analyzing all samples, their experimental/expected results, and the ability to detect
mutations and drug-resistant calls in all four drug-resistant regions of the Pol Region (NRTI, NNRTI, PI, and INSTI). Table 6 shows a 100% concordance between expected and experimental results for
viral copy number dilutions up to 10^ (-5) or 488 copies.

At 10^ (-6) or 49 viral copies, sensitivity decreases to 8/12 = 67%.

These results in Table 7 indicate that positive HIV-1 samples with viral loads up to 488 copies can reliably produce a drug resistance profile. Viral copies under 488 have inconsistent results and do not pass quality control metrics.

Table 1A. HIV-1 Positive Control Quality Control Metrics (Median) – Triplicate Average Mean*

Number of Copies	Dilution	Assembly Length Unambiguous	Percent Reference Coverage	Assembly Mean Coverage	Number of Ns	Total Number
4,880,000	HIV_PC_1:10	9053	98.6	8050	117	9177
488,000	HIV_PC_1:10e2	9040	98.5	5620	116	9163
48,800	HIV_PC_1:10e3	9040	98.5	6291	110	9157
4,880	HIV_PC_1:10e4	7608 (*8907)	82.9 (*97.0)	9096 (*9391)	1307 (*251)	8919 (*9163)
488	HIV_PC_1:10e5	8532	92.4	4760	608	9149
48.8	HIV_PC_1:10e6	6496	70.8	2215	2651	9151

*One sample from HIV_PC_1:10e4 (2) failed QC due to sample loss during library preparation. Analysis shows inclusion and omission metrics. Triplicate Average: Average mean calculation for dilutions (all dilutions were run in triplicate)

Table 1B. HIV-1 Positive Control Quality Control Metrics (Raw Data) Analysis on Terra.bio

	A	B	C	D	E	F
	Dilution Factor	**Assembly Length Unambiguous**	**Assembly Mean Coverage**	**Number of Ns**	**Total Number**	**% Reference Coverage**

	A	B	C	D	E	F
	HIV_PC_10_1	9040	6854.19	128	9175	98.46
	HIV_PC_10_2	9040	5455.84	135	9182	98.46
	HIV_PC_10_3	9079	11842.6	88	9174	98.89
	HIV_PC_100_1	9040	6085.74	125	9172	98.46
	HIV_PC_100_2	9041	6447.61	122	9169	98.48
	HIV_PC_100_3	9040	4327.41	102	9149	98.46
	HIV_PC_1000_1	9041	10369.2	105	9152	98.48
	HIV_PC_1000_2	9040	4998.39	121	9168	98.46
	HIV_PC_1000_3	9039	3504.9	105	9152	98.45
	HIV_PC_10000_1	9044	14738.5	127	9174	98.51
	HIV_PC_10000_2	***5010***	***8504.21***	***3419***	***8432***	***54.57***
	HIV_PC_10000_3	8769	4044.16	375	9151	95.51
	HIV_PC_100000_1	8739	5186.66	399	9142	95.19
	HIV_PC_100000_2	8730	5353.17	411	9156	95.09
	HIV_PC_100000_3	8129	3741.45	1014	9149	88.54
	HIV_PC_1000000_1	6263	2370.05	2879	9147	68.22
	HIV_PC_1000000_2	6784	2204.5	2361	9151	73.89
	HIV_PC_1000000_3	6441	2068.95	2713	9156	70.16

*One sample from HIV_PC_1:10e4 [HIV_PC_10000_2] failed QC due to sample loss during library preparation.

Table 3. HIV Drug Resistance Mutations

HIV-1 drug-resistant profiles from Exatype NGS, using the Stanford HIV drug-resistant database.

	A	B	C	D	E
	Dilution Factor	**NRTI – Nucleoside Reverse Transcriptase Inhibitors**	**NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitors**	**PI - Protease Inhibitors**	**INSTI - Integrase Inhibitors**
	HIV_PC_10_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10_2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10_3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100_2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100_3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_1000_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_1000_2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible

	A	B	C	D	E
	HIV_PC_1000_3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10000_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10000_2	***Low coverage***	***Low coverage***	***Low coverage***	***Low coverage***
	HIV_PC_10000_3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100000_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100000_2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_100000_3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_1000000_1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Low coverage
	HIV_PC_1000000_2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Low coverage	Low coverage
	HIV_PC_1000000_3	Susceptible	2-Susceptible, 3-Low coverage ETR : [RT] F227L	Susceptible	Low coverage

*One sample from HIV_PC_1:10e4 [HIV_PC_10000_2] failed QC due to sample loss during library preparation.

Table 4: Within Run Precision

	A	B	C	D	E	F
	Sensitivity	**Number of Copies n=6/dilution**	**NRTI – Nucleoside Reverse Transcriptase Inhibitors**	**NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitors**	**PI - Protease Inhibitors**	**INSTI - Integrase Inhibitors**
	8/12	HIV_PC_10^(-6), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Low coverage
	HIV_PC_10^(-6), _2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Low coverage	Low coverage	Low coverage
	HIV_PC_10^(-6), _3	Susceptible	2-Susceptible, 3-Low coverage ETR : [RT] F227L	Susceptible	Low coverage	Low coverage
	12/12	HIV_PC_10^(-5), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10^(-5), _2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	HIV_PC_10^(-5), _3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible

	A	B	C	D	E	F
	*8/12 8/8	HIV_PC_10^(-4), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10^(-4), _2	Low coverage	Low coverage	Low coverage	Low coverage	Susceptible
	HIV_PC_10^(-4), _3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	12/12	HIV_PC_10^(-3), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10^(-3), _2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	HIV_PC_10^(-3), _3	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	12/12	HIV_PC_10^(-2), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10^(-2), _2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	HIV_PC_10^(-2), _3	Susceptible	2-Susceptible, 3-Intermediate	Susceptible	Susceptible	Susceptible

	A	B	C	D	E	F
			ETR : [RT] F227L			
	12/12	HIV_PC_10^(-1), _1	Susceptible	2- Susceptible, 3- Intermediate ETR : [RT] F227L	Susceptible	Susceptible
	HIV_PC_10^(-1)' _2	Susceptible	2- Susceptible, 3- Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible
	HIV_PC_10^(-1)' _3	Susceptible	2- Susceptible, 3- Intermediate ETR : [RT] F227L	Susceptible	Susceptible	Susceptible

(64/68) x 100 = 94%
*(64/72) x 100 = 89%
*One sample from HIV_PC_1:10e4 [HIV_PC_10000_2] failed QC due to sample loss during library preparation.

Table 5: Analytical Specificity for Mutation Detected:

	A	B	C
	Analytical Specificity	**Number of Copies n=6/dilution**	**NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitors**
	3/3	HIV_PC_10^(-6)	ETR : [RT] F227L
	3/3	HIV_PC_10^(-5)	ETR : [RT] F227L
	*2/3 2/2	HIV_PC_10^(-4)	ETR : [RT] F227L
			Low coverage
			ETR : [RT] F227L

	A	B	C
	3/3	HIV_PC_10^(-3)	ETR : [RT] F227L
	3/3	HIV_PC_10^(-2)	ETR : [RT] F227L
	3/3	HIV_PC_10^(-1)	ETR : [RT] F227L

(17/17) x 100 =100.00%
*(17/18) x 100 = 94%
*One sample from HIV_PC_1:10e4 (2) failed QC due to sample loss during library preparation. Analysis shows inclusion and omission metrics.

Table 6: Analytical Specificity of Co-infection

	A	B	C
	Accession #	**Specimen**	**HIV-1 Assay Result**
	7427	HAV+	No sequence
	1701	HBV+	No sequence
	1740	HCV+	No sequence
	4023	TP+	No sequence
	7214	TP+	No sequence

Table 7: Limit of Detection Threshold

	A	B	C	D	E	F
	Sensitivity	**Number of Copies n=6/dilution**	**NRTI – Nucleoside Reverse Transcriptase Inhibitors**	**NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitors**	**PI - Protease Inhibitors**	**INSTI - Integrase Inhibitors**
	8/12	HIV_PC_10^(-6), _1	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Susceptible	Low coverage

	A	B	C	D	E	F
	HIV_PC_10^(-6), _2	Susceptible	2-Susceptible, 3-Intermediate ETR : [RT] F227L	Low coverage	Low coverage	Low coverage
	HIV_PC_10^(-6), _3	Susceptible	2-Susceptible, 3-Low coverage ETR : [RT] F227L	Susceptible	Low coverage	Low coverage

At 10^ (-6) or 49 viral copies, sensitivity decreases to 8/12 = 67%.

These results indicate that positive HIV-1 samples with viral loads up to 488 copies can reliably produce a drug resistance profile. However, viral copies under 488 have inconsistent results and do not pass quality control metrics.

Note:

It is important to note that the positive control was a culture of a USA HIV-1 strain IIIB, and primers used for this assay were designed based on HIV-1 sequences primarily from North America. Due to the high genome variability of
the HIV-1 virus, primers must be designed using sequences in or around the region of interest. The primers used in this assay performed well, with good genome coverage on samples from North America. However, they showed poor coverage of HIV-1-positive samples from different continents. Hence, the recommendation of designing primers specific to the region to account for variations within the region to achieve higher genome coverage.

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immunodeficiency virus drug resistance: 2018 recommendations of the international antiviral society-USA panel. *Clin. Infect. Dis.* 2019;**68**:177–187. doi: 10.1093/cid/ciy463

[4] "**Illumina COVIDSeq RUO Kits Reference Guide (1000000126053 v08)**" *Illumina COVIDSeq Research Use Only Kits Documentation.* **COVIDSeq Assay (96 samples) | Low-throughput COVID-19 surveillance (illumina.com)**. 2022

Guidelines

Follow the manufacturer's specifications and guidelines.

Materials

HIV 1 Primers:

	A	B	C
	Well Position	Name	Sequence
	A1	HIV-1_v1.0_1_LEFT	TGGTTAGACCAGATCTGAGCCT
	A2	HIV-1_v1.0_1_RIGHT	TTTCTTTCCCCCTGGCCTTAAC
	A3	HIV-1_v1.0_3_LEFT	GCTTTAGACAAGATAGAGGAAGAGCA
	A4	HIV-1_v1.0_3_RIGHT	TTCCTGCTATGTCACTTCCCCT
	A5	HIV-1_v1.0_5_LEFT	TTGGATGACAGAAACCTTGTTGG
	A6	HIV-1_v1.0_5_RIGHT	AAGAAAATTCCCTGGCCTTCCC
	A7	HIV-1_v1.0_7_LEFT	ATTAGAAGAAATGAGTTTGCCAGGAA
	A8	HIV-1_v1.0_7_RIGHT	TTCTTTATGGCAAATACTGGAGTATTGT
	A9	HIV-1_v1.0_9_LEFT	GAGACACCAGGGATTAGATATCAGT
	A10	HIV-1_v1.0_9_RIGHT	CCCTGGGTAAATCTGACTTGCC
	A11	HIV-1_v1.0_11_LEFT	AGAGCCATTTAAAAATCTGAAAACAGGA
	A12	HIV-1_v1.0_11_RIGHT	CAGTCTTCTGATTTGTTGTGTCAGT
	B1	HIV-1_v1.0_13_LEFT	GTCAGTGCTGGAATCAGGAAAGT
	B2	HIV-1_v1.0_13_RIGHT	CGTAGCACCGGTGAAATTGCT
	B3	HIV-1_v1.0_15_LEFT	AGACATAATAGCAACAGACATACAAACT
	B4	HIV-1_v1.0_15_RIGHT	CCAATCTAGCATCCCCTAGTGG
	B5	HIV-1_v1.0_17_LEFT	CAAGCAGGACATAACAAGGTAGGA
	B6	HIV-1_v1.0_17_RIGHT	TCCAGGGCTCTAGTCTAGGATC
	B7	HIV-1_v1.0_19_LEFT	TCTCTATCAAAGCAGTAAGTAGTACATGT
	B8	HIV-1_v1.0_19_RIGHT	GCATGTGTGGCCCAAACATTAT
	B9	HIV-1_v1.0_21_LEFT	AGCGGGAGAATGATAATGGAGAA
	B10	HIV-1_v1.0_21_RIGHT	GCATTGTCCGTGAAATTGACAGA
	B11	HIV-1_v1.0_23_LEFT	AGCTAGCAAATTAAGAGAACAATTTGGA

	A	B	C
	B12	HIV-1_v1.0_23_RIGHT	TTCACTTCTCCAATTGTCCCTCA
	C1	HIV-1_v1.0_25_LEFT	CTATTGAGGCGCAACAGCATCT
	C2	HIV-1_v1.0_25_RIGHT	ACCTACCAAGCCTCCTACTATCA
	C3	HIV-1_v1.0_27_LEFT	ACCACCGCTTGAGAGACTTACT
	C4	HIV-1_v1.0_27_RIGHT	TGCTCCATGTTTTTCCAGGTCT
	C5	HIV-1_v1.0_29_LEFT	CACACACAAGGCTACTTCCCTG
	C6	HIV-1_v1.0_29_RIGHT	AACCAGAGAGACCCAGTACAGG
	F1	HIV-1_v1.0_2_LEFT	TAGAAGGAGAGAGATGGGTGCG
	F2	HIV-1_v1.0_2_RIGHT	TTTTGGCTGACCTGATTGCTGT
	F3	HIV-1_v1.0_4_LEFT	GCTGCAGAATGGGATAGAGTGC
	F4	HIV-1_v1.0_4_RIGHT	TTCTTCTAGTGTAGCCGCTGGT
	F5	HIV-1_v1.0_6_LEFT	GGAAGGACACCAAATGAAAGATTGT
	F6	HIV-1_v1.0_6_RIGHT	TGTCCACAGATTTCTATGAGTATCTGA
	F7	HIV-1_v1.0_8_LEFT	AGTAGAAATTTGTACAGAGATGGAAAAGG
	F8	HIV-1_v1.0_8_RIGHT	AAGGCTCTAAGATTTTTGTCATGCT
	F9	HIV-1_v1.0_10_LEFT	CAGCCTATAGTGCTGCCAGAAA
	F10	HIV-1_v1.0_10_RIGHT	TTTGCACTGCCTCTGTTAATTGT
	F11	HIV-1_v1.0_12_LEFT	GGGAGACTAAATTAGGAAAAGCAGGA
	F12	HIV-1_v1.0_12_RIGHT	AGCCATTGCTCTCCAATTACTGT
	G1	HIV-1_v1.0_14_LEFT	GGGCAGGAAACAGCATATTTTCT
	G2	HIV-1_v1.0_14_RIGHT	TGCTGTCCCTGTAATAAACCCG
	G3	HIV-1_v1.0_16_LEFT	GGGAAAGCTAGGGGATGGTTTT
	G4	HIV-1_v1.0_16_RIGHT	TCGTAACACTAGGCAAAGGTGG
	G5	HIV-1_v1.0_18_LEFT	GCAACAACTGCTGTTTATCCATTTT
	G6	HIV-1_v1.0_18_RIGHT	TTTCCTATATTCTATGATTACTATGGACCAC
	G7	HIV-1_v1.0_20_LEFT	TACCTGTGTGGAAGGAAGCAAC
	G8	HIV-1_v1.0_20_RIGHT	TGCATATTCTTTCTGCACCTTACCT

	A	B	C
	G9	HIV-1_v1.0_22_LEFT	GCCAGTAGTATCAACTCAACTGCT
	G10	HIV-1_v1.0_22_RIGHT	ACAGTAGAAAAATTCCCCTCCACA
	G11	HIV-1_v1.0_24_LEFT	GGGCTGCTATTAACAAGAGATGGT
	G12	HIV-1_v1.0_24_RIGHT	AGGTATCTTTCCACAGCCAGGA
	H1	HIV-1_v1.0_26_LEFT	TGGGCAAGTTTGTGGAATTGGT
	H2	HIV-1_v1.0_26_RIGHT	ACCAATATTTGAGGGCTTCCCAC
	H3	HIV-1_v1.0_28_LEFT	TGGATGGCCTACTGTAAGGGAA
	H4	HIV-1_v1.0_28_RIGHT	AGCTTGTAGCACCATCCAAAGG

Troubleshooting

1 Sequencing libraries were prepared using reagents in the Illumina COVIDSeq Assay (RUO) kit with a protocol modified by substituting SARS-CoV-2 primers with HIV-1 primers.

2 **Sample Extraction**

HIV-1 subtype B positive culture control ZeptoMetrix (Part #v0801032CF), was extracted via the Qiagen Advanced XL DSP kit on the EZ1 extractor and serially diluted to the following concentration: 10^(-1), 10^(-2), 10^(-3), 10^(-4), 10^(-5), and 10^(-6), Undiluted control (1.74*10^10 IU/mL or 2.09*10^10 ng/ml); Sample was diluted 1:10 = 2.09*10^9 ng/ml before extraction. **cDNA Synthesis and Amplification**

3 Prepare the HIV-1 primer pool as described by the manufacturer. Individual primers were obtained from IDT as desalted, lab-ready stock at 100mM concentration. Dilute each primer at ratios provided by the manufacturer. Dilute the primer pool to get a 10 µM working concentration.

4 Prepare the following consumables:

	Reagent	Storage	Instructions
	EPH3	-25°C to -15°C	Thaw at room temperature, and then invert to mix

Save the following COVIDSeq Anneal program on the thermal cycler:

- Choose the preheat lid option
- Set the reaction volume to 17 µl
- 65°C for 3 minutes
- Hold at 4°C

Procedure

1. Label new PCR plate CDNA1.
2. Add 8.5 µl EPH3 to each well.
3. Add 8.5 µl eluted sample to each well.
4. Seal and shake at 1600 rpm for 1 minute.
5. Centrifuge at 1000 × g for 1 minute.
6. Place on the preprogrammed thermal cycler and run the COVIDSeq Anneal program.

5 **Synthesize First Strand cDNA:**

	A	B	C
	Reagent	**Storage**	**Instructions**
	RVT	-25°C to -15°C	Invert to mix before use. Keep on ice
	FSM	-25°C to -15°C	Thaw and bring to room temperature. Invert to mix, and then keep on ice.

Save the following COVIDSeq FSS program on the thermal cycler:

- Choose the preheat lid optio.n
- Set the reaction volume to 25 µl
- 25°C for 5 minutes
- 50°C for 10 minutes
- 80°C for 5 minutes
- Hold at 4°C

Procedure

1. In a 1.7 ml tube, combine the following volumes to prepare First Strand cDNA Master Mix.
   Multiply each volume by the number of samples.
- FSM (9 µl)
- RVT (1 µl)

Reagent overage is included to account for small pipetting errors.
2. Add 8 µl master mix to each well of the CDNA1 plate.
3. Seal and shake at 1600 rpm for 1 minute.
4. Centrifuge at 1000 × g for 1 minute.
5. Place on the preprogrammed thermal cycler and run the COVIDSeq FSS program

SAFE STOPPING POINT: If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

6 **Amplify cDNA:**

Preparation:

1. Prepare the following consumables:

	A	B	C
	Reage	**Storage**	**Instructions**

	A	B	C
	nt		
	CPP1	-25°C to -15°C	Thaw at room temperature. Keep on ice until use.
	CPP2	-25°C to -15°C	Thaw at room temperature. Keep on ice until use.
	IPM	-25°C to -15°C	Thaw at room temperature, and then invert to mix. Keep on ice until use.

2. Save the following COVIDSeq PCR program on the thermal cycler:

- Choose the preheat lid option
- Set the reaction volume to 25 µl
- 98°C for 3 minutes
- 35 cycles of:
  98°C for 15 seconds
  63°C for 5 minutes
- Hold at 4°C

Procedure:

1. Label two new PCR plates COV1 and COV2. The plates represent two separate PCR reactions on
each sample and control in the CDNA1 plate.

2. In a 15 ml tube, combine the following volumes to prepare COVIDSeq PCR 1 Master Mix and
COVIDSeq PCR 2 Master Mix. Multiply each volume by the number of samples. Reagent overage is included to account for small pipetting errors.

	A	B	C
	Reagent	COVIDSeq PCR 1 Master Mix (µl)	COVIDSeq PCR 2 Master Mix (µl)
	IPM	15	15
	CPP1	4.3	N/A
	CPP2	N/A	4.3
	Nuclease-free water	4.7	4.7

3. Add 20 µl COVIDSeq PCR 1 Master Mix to each well of the COV1 plate corresponding to each well of the CDNA1 plate.

4. Add 5 µl first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the COV1 plate.
5. Add 20 µl COVIDSeq PCR 2 Master Mix to each well of the COV2 plate corresponding to each well of the CDNA1 plate.
6. Add 5 µl first strand cDNA synthesis from each well of the CDNA1 plate to the corresponding well of the COV2 plate.
7. Seal and shake at 1600 rpm for 1 minute.
8. Centrifuge at 1000 x g for 1 minute.
9. Place in the preprogrammed thermal cycler and run the COVIDSeq PCR program.

SAFE STOPPING POINT If you are stopping, seal the plate and store at -25°C to -15°C for up to 3 days.

7 **Tagment PCR Amplicons**

Preparation:

1. Prepare the following consumables:

	A	B	C
	Reagent	**Storage**	**Instructions**
	EBLTS	2°C to 8°C	Bring to room temperature. Vortex thoroughly before use.
	TB1	-25°C to -15°C	Bring to room temperature. Vortex thoroughly before use.

2. If COV1 and COV2 plates were stored frozen, prepare as follows.
a. Thaw at room temperature.
b. Check seals, and then shake at 1600 rpm for 1 minute.
c. Centrifuge at 1000 x g for 1 minute.

3. Save the following COVIDSeq TAG program on the thermal cycler:
- Choose the preheat lid option
- Set the reaction volume to 50 µl
- 55°C for 5 minutes
- Hold at 10°C

Procedure:

1. Label a new PCR plate TAG1.
2. Combine COV1 and COV2 as follows.

a. Transfer 10 µl from each well of the COV1 plate to the corresponding well of the TAG1 plate.

b. Transfer 10 µl from each well of the COV2 plate to each well of the TAG1 plate containing COV1.

3. In a 15 ml tube, combine the following volumes to prepare Tagmentation Master Mix. Multiply each volume by the number of samples.

- TB1 (12 µl)
- EBLTS (4 µl)
- Nuclease-free water (20 µl)

4. Add 30 µl master mix to each well in TAG1 plate.
5. Seal and shake at 1600 rpm for 1 minute.
6. Place on the preprogrammed thermal cycler and run the COVIDSeq TAG program.

8 **Post Tagmentation Clean Up:**

Preparation:

1. Prepare the following consumables:

	A	B	C
	Reagent	**Storage**	**Instructions**
	ST2	Room temperature	Vortex before use.
	TWB	2°C to 8°C	Vortex before use.

Procedure:

1. Centrifuge the TAG1 plate at 500 x g for 1 minute.
2. Add 10 µl ST2 to each well of the TAG1 plate.
3. Seal and shake at 1600 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge at 500 × g for 1 minute.
6. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
7. Inspect for bubbles on the seal. If present, centrifuge at 500 x g for 1 minute, and then place on the magnetic stand (~3 minutes).
8. Remove and discard all supernatant
9. Wash beads as follows.
   a. Remove from the magnetic stand.
   b. Add 100 µl TWB to each well.
   c. Seal and shake at 1600 rpm for 1 minute.
   d. Centrifuge 500 × g for 1 minute.
   e. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).

f. For first wash only, remove and discard all supernatant from each well.

10. Wash beads a second time. Leave supernatant in plate for second wash to prevent beads from overdrying.

9 **Amplify Tagmented Amplicons:**

Preparation

1. Prepare the following consumables:

	A	B	C
	Reagent	**Storage**	**Instructions**
	EPM	-25°C to -15°C	Invert to mix. Keep on ice until use
	Index adapters	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge at 1000 × g for 1 minute.

2. Open each prepared index adapter plate seal as follows. Use a new PCR plate for each different index set.

a. Align a new 96-well PCR plate above the index adapter plate, and then press down to puncture the foil seal.
b. Discard the PCR plate.

3. Save the following COVIDSeq TAG PCR program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- Set the reaction volume to 50 µl
- 72°C for 3 minutes
- 98°C for 3 minutes
- 7 cycles of:
  - 98°C for 20 seconds
  - 60°C for 30 seconds
  - 72°C for 1 minute
  - 72°C for 3 minutes
- Hold at 10°C

Procedure:

1. In a 15 ml tube, combine the following volumes to prepare PCR Master Mix. Multiply each volume by the number of samples.
   - EPM (24 µl)
   - Nuclease-free water (24 µl)
2. Vortex PCR Master Mix to mix.
3. Keep the TAG1 plate on magnetic stand and remove TWB.
4. Use a 20 µl pipette to remove any remaining TWB.
5. Remove the TAG1 plate from the magnetic stand.
6. Add 40 µl PCR Master Mix to each well.
7. Add 10 µl index adapters to each well of the PCR plate.
8. Seal and shake at 1600 rpm for 1 minute.
9. If liquid is visible on the seal, centrifuge at 500 x g for 1 minute.
10. Inspect to make sure beads are resuspended. To resuspend, set your pipette to 35 µl with the plunger down, and then slowly pipette to mix.
11. Place on the preprogrammed thermal cycler and run the COVIDSeq TAG PCR program.

10 **Pool and Clean Up Libraries**

Preparation:

1. Prepare the following consumables:

	A	B	C
	Reagent	**Storage**	**Instructions**
	ITB or IPB	Room temperature	Vortex thoroughly to mix.
	RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex and invert to mix

2. Prepare 2.5 ml 80% EtOH from absolute EtOH for each tube of pooled libraries.

Procedure for Illumina COVIDSeq Assay (96 Samples):

1. Centrifuge the TAG1 plate at 500 × g for 1 minute.
2. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
3. To pool libraries, complete the following steps appropriate for your kit. Repeat the steps for each additional sample plate.
   a. Label a new 1.7 ml tube Pooled IPB. b
   b. Transfer 5 µl library from each well of the TAG 1 plate into the Pooled IPB tube.

4. Vortex the Pooled IPB tubes to mix, and then centrifuge briefly.

5. Vortex IPB to resuspend.
6. Add IPB using the resulting volume of Pooled IPB tube volume multiplied by 0.9. For example, for 96 samples, add 432 µl IPB to each tube.

7. Vortex to mix.
8. Incubate at room temperature for 5 minutes.
9. Centrifuge briefly.
10. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
11. Remove and discard all supernatant.
12. Wash beads as follows.
a. Keep on the magnetic stand and add 1000 µl fresh 80% EtOH to each tube.
b. Wait 30 seconds.
c. Remove and discard all supernatant.

13. Wash beads a second time.
14. Use a 20 µl pipette to remove all residual EtOH.
15. Add 55 µl RSB.
16. Vortex to mix, and then centrifuge briefly.
17. Incubate at room temperature for 2 minutes.
18. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. Transfer 50 µl supernatant from each Pooled IPB tube to a new microcentrifuge tube.

SAFE STOPPING POINT If you are stopping, cap the tube and store at -25°C to -15°C for up to 30 days.

11 **Quantify and Normalize Libraries**

1. Analyze 2 µl library pool using a Qubit dsDNA HS Assay kit. If libraries are outside the standard range, dilute to 1:10 concentration and analyze again.

2. Dilute each library pool to a minimum of 30 µl at a normalized concentration 4 nM using RSB according to manufacture specification.

Pool and Dilute Libraries:

After diluting to the starting concentration of 4 nM, libraries are ready to be denatured and diluted
to the final loading concentration according to the specifications of the Illumina sequencing platform.

Protocol references

[4] "**Illumina COVIDSeq RUO Kits Reference Guide (1000000126053 v08)**" *Illumina COVIDSeq Research Use Only Kits Documentation.* **COVIDSeq Assay (96 samples) | Low-throughput COVID-19 surveillance (illumina.com)**. 2022