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Next Generation Sequencing of HIV-1 Drug Resistant Mutations V.2

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HIV

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The Florida Department of Health's Bureau of Public Health Laboratories in Jacksonville has developed a protocol for the Next Generation Sequencing (NGS) of HIV, primarily for the purpose of drug-resistant mutation identification. This HIV-1 protocol uses amplicon-based sequencing based on primers designed by the BEEHIVE Consortium (https://www.beehive.ox.ac.uk/). The amplified pol gene regions can be used in both genotyping and drug resistance determination. Our protocol utilizes newer enzymes with higher fidelity for sequencing and Illumina sequencing technology. We have cross verified 3 different Illumina Sequencing platforms to ensure that all produce equivalent results so that in the event of a surge samples can be sequenced quickly and in mixed-species pools.
The NGS data generated can also be used in surveillance and outbreak monitoring, giving epidemiologist more information about circulating viral genomes. There is also the potential that this protocol can be expanded to whole genome sequencing for HIV-1.
The imminent sunsetting of ViroSeq (Abbott Molecular) has required many labs to look for new methods to continue identifying HIV-1 drug resistance strains for both clinical management and epidemiological study. NGS was chosen as it is more cost effective than investing in a single pathogen platform. NGS allows for one sample to produce results and data that can aid not just a patient but an entire population.

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Correction to amplicon PCR

HIV-1, Drug resistance, Drug resistance mutations, Next generation sequencing, SmartGene, FLDOH, Retrovirus sequencing, HIV, public health, antiretroviral drugs, HIV-1 clinical test

protocol ,

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All Lab Developed Tests are still subject to CLIA. Please consult with your CLIA director to establish an appropriate study to develop your own HIV-1 sequencing test.

QIAmp Viral RNA Mini Kit (RUO or DSP) (Qiagen 52904/61904
MagMax Viral/Pathogen II MVPII Nucleic Acid Isolation Kit (Thermofisher A48383)
SSIV VILO Master Mix (Thermofisher, Cat 11756050)
Q5 Master Mix (NEB, Cat M0492S)
Primers (Gall A, et al. Journal of Clinical Microbiology. 2012; 50:12)

Set and primer	Sequence (5'–3')	PositionsaProduct sizea 2
Pan-HIV-1_2F	GGG AAG TGA YAT AGC WGG AAC	1031–1051
3,574 bp Pan-HIV-1_2R	CTG CCA TCT GTT TTC CAT ART C	4604–4583
3 Pan-HIV-1_3F	TTA AAA GAA AAG GGG GGA TTG GG	4329–4351
3,066 bp Pan-HIV-1_3R	TGG CYT GTA CCG TCA GCG	7394–7377

According to HIV-1 reference strain HXB2 (GenBank accession number NC001802).

Single/multichannel pipettes with p20/p200/p1000 tips
Thermocycler
Nuclease-free water
AMPure XP Beads (Beckman Coulter)
Magnetic stand
Tapestation or Agarose gel
Qubit or other quantitation method
Illumina Nextera XT DNA Library Prep Kit
Illumina Nextera v2 Index Kits
Illumina iSeq
Illumina iSeq 100 i1 v2 cartridge
Illumina MiSeq
Illumina NextSeq
https://www.smartgene.com/
Pipeline: HIV1-PR+RT+IN (2.4.5_HIV1_v1.6)

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We are happy to share HIV-1 samples for public health lab validations if we have materials available.

RNA Extraction

1 RNA Extraction has been verified using the following methods

1.1 Qiagen QIAmp Viral RNA Mini Kit (DSP or RUO)
https://www.qiagen.com/us/products/diagnostics-and-clinical-research/sample-processing/qiaamp-viral-rna-kits/

1.2 Thermofisher MagMAX Viral/Pathogen II (MVP II) Nucleic Acid Isolation kit
https://www.thermofisher.com/order/catalog/product/A48383

cDNA Synthesis

2 Master Mix
- **4.0 µL** SuperScript IV VILO Master Mix
- **6.0 µL** Nuclease Free Water
- **10.0 µL** RNA template

3 Run the following protocol on a thermocyler 25m
- **25 °C** **00:10:00**
- **50 °C** **00:10:00**
- **85 °C** **00:05:00**

Amplicon PCR

4 Each fragment will need to be amplified in an individual PCR reaction
Set 1
Pan-HIV-1_2F GGG AAG TGA YAT AGC WGG AAC
Pan-HIV-1_2R CTG CCA TCT GTT TTC CAT ART C

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Set 2
Pan-HIV-1_3F TTA AAA GAA AAG GGG GGA TTG GG
Pan-HIV-1_3R TGG CYT GTA CCG TCA GCG

4.1 Master Mix

- **12.5 µL** 2x Q5 Master Mix
- **0.5 µL** Forward Primer20 Micromolar (µM)
- **0.5 µL** Reverse Primer20 Micromolar (µM)
- **6.5 µL** Nuclease Free Water
- **5.0 µL** cDNA template

7m 15s

5 PCR
The two primers do have different optimal annealing temperatures, but we have found that they both can be run at the same temperature.

- **105 °C** Lid
- **50 °C** **00:00:30**
- **98 °C** **00:00:30**

40 cycles-
- **98 °C** **00:00:15**
- **55 °C** **00:00:30**
- **98 °C** **00:00:30**

- **72 °C** **00:05:00**
- **4 °C** Hold

6 Bead clean up using a ratio of 0.5- follow the AMPure XP bead protocol for PCR purification.

7 Check fragment on Tapestation or gel.

Band size should be
Amplicon 1- 3.5 kB
Amplicon 2- 3.0 kB

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Sample Amplicon Pooling

8 Sample fragments 1 and 2 can be pooled in eqimolar amounts or in equal concentrations.

For HIVdb version9.0(last updated on2021-02-22) Primer Set 1 usually has sufficient coverage. Primer Set 2 offers end coverage.

9 Pool fragments

10 Dilute as needed to achieve **1.0 ng** input concentration for library preparation

Library Prep

11 Follow Illumina Protocol for Nextera XT DNA Library Sample Prep

Library Pooling

12 Amplicon quality can effect how many samples can be pooled onto one run. Use caution in deciding how many samples to pool.

Sequencing

13 We have successfully sequenced these libraries on the following platforms:

iSeq
MiSeq
NextSeq

The MiSeq and NextSeq are usually mixed organism pools. This has had no discernable adverse effect on HIV-1 Drug-Resistance Sequencing results.

Analysis

14 We currently use SmartGene HIV-1 pipeline (https://www.smartgene.com/) for analysis

Pipeline Name: HIV-1 PR+RT+IN
Version 2.4.5_HIV1_v1.6
Noise Filter [%] 0.5
Interpretation cut off [%] 5.0

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Minimum read depth and additional criteria should be determined by your institution

References

15 Gall A, Ferns B, Morris C, Watson S, Cotten M, Robinson M, Berry N, Pillay D, Kellan P. Universal Amplification, Next-Generation Sequencing, and Assembly of HIV-1 Genomes. Journal of Clinical Microbiology. 2012; 50:12.
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Previous Protocols
https://dx.doi.org/10.17504/protocols.io.btrnnm5e
https://dx.doi.org/10.17504/protocols.io.btpqnmmw

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