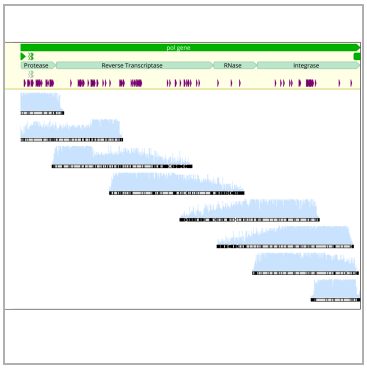

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Affordable method for genotyping HIV-1 reverse transcriptase, protease and integrase genes: an in-house protocol

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ABSTRACT

HIV drug resistance (HIVDR) remains a major threat to achieving sustainable viral suppression on antiretroviral treatment. To overcome concerns over increasing levels in pretreatment drug resistance and subsequent cost implications, most countries including those in resource limited settings (RLS) adopted use of dolutegravir (DTG), a potent integrase strand transfer inhibitor (INSTI). As more people receive INSTIs, the demand for INSTI resistance testing is increasing. Current HIVDR testing methods in RLS focus on genotyping HIV protease (PR) and reverse transcriptase (RT) genes, separate from the integrase (IN) gene. However, such an approach is expensive and evidently increases the workload for HIVDR genotyping. As a result, affordable and labour efficient methods that genotype all relevant HIV-1 genes (i.e., the PR, RT and IN genes) are required to guide clinical decisions, especially in RLS where cost is a major limiting factor. Therefore, we developed a protocol for genotyping complete HIV-1C genes at an affordable cost and time-efficient manner, that can be adopted for use in HIV drug resistance genotyping. The main limitation to this protocol is that it was only tested on HIV-1C samples, and thus might not be effective against other HIV subtypes. In conclusion, the expected results of using this protocol include complete HIV-1C sequence coverage of all relevant viral gene regions; i.e. PR codons 1 - 99, RT codons 1 - 560, and IN codons 1 - 288, for HIVDR genotyping.

Protocol status: Working

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PROTOCOL integer ID: 58965

Keywords: HIV-1, pol gene, resource limited settings, genotyping, HIV drug resistance

GUIDELINES

Introduction

This protocol describes a simple, labour efficient and affordable genotyping method for detecting HIVDR mutations in the HIV-1C protease, reverse transcriptase and integrase genes. This method was produced at the Department of Virology National Laboratory Services, University of KwaZulu-Natal, in Durban, South Africa.

Briefly, a simple and innovative single assay polymerase chain reaction (PCR) technique was designed to amplify an approximate 2.9kb HIV-1 pol gene. The method was designed to genotype HIV-1 pol genes with complete coverage of all regions with known HIV drug resistance mutations by overlapping 8 sequencing primers. The amplicons generated are sequenced on an ABI Genetic Analyzer, with HIVDR mutations determined using the Stanford HIV drug resistance database.

Purpose

The purpose of this protocol is to provide detailed instructions that should be followed when genotyping HIV-1 pol genes from plasma samples using BigDye Terminator v3.1 cycle sequencing kit (LifeTechnologies) and Sanger sequencing.

Competing Interests

There are no competing interests to declare.

Data availability

https://www.ncbi.nlm.nih.gov/nuccore/OM468298

MATERIALS

LIST OF MATERIALS USED WITH CATALOGUE NUMBERS

Extraction

- NucliSENS easyMAG consumables - 280135 & 280133

PCR

- SuperScript IV One-Step PCR reagents - 12594100
- Platinum Taq reagents - 10966018
- dNTP Mix (10mM each) - R0191

Gel electrophoresis

- TopVision Agarose Tablets - R2801
- UltraPure TBE Buffer, 10X - 15581044
- Orange DNA Loading Dye (6X) - R0631
- SYBR Safe DNA Gel Stain - S33102
- GeneRuler 1 kb DNA Ladder - SM0314

Product Purification

- ExoSAP-IT purification kit - 75001.1.EA

Sequencing

- BigDye Terminator v3.1 Cycle Sequencing kit - 4337455
- BigDye XTerminator Purification kit - 4376487

Consumables

- Tips
- MicroAmp 8-Tube Strip - A30589
- Adhesive PCR Plate Foils (nuclease-free) - AB0626
- MicroAmp Optical 96-Well Reaction Plate - N8010560
- Nuclease-free Water - AM9932
- 1.5ml nuclease free eppendorf tubes - AM12400
- Personal protective equipment

Instruments and software

- Pipettes
- Refrigerated microcentrifuge
- Biosafety cabinets
- Vortexer
- NucliSENS easyMAG
- Mini-centrifuge
- Thermal cycler
- Gel electrophoresis unit
- UV detection unit
- Plate centrifuge
- Genetic Analyzer
- Geneious software
- Stanford HIV drug resistance database

SAFETY WARNINGS

The use of potentially infectious material requires that all persons must use laboratory safety protective clothing. It is advisable for all users to have undergone safety training to ensure safe handling and disposal of harmful material. The disinfection of work areas is required before and after work.

BEFORE START INSTRUCTIONS

- Ensure that the working areas are disinfected
- Remember to check the expiring date of the reagents before use
- Ensure that instruments maintenance is up to date

EXTRACTION

1 Sample preparation and RNA extraction on NucliSENS easyMAG

1.1 Retrieve plasma samples from -80 °C freezer and leave to equilibrate to room temperature prior to processing.

1.2 Transfer 500 µL of plasma into well labeled 1.5 mL Sarstedt screw-cap tubes/ eppendorf tubes and place in a pre-cooled microcentrifuge at 4 °C.

1.3 Mark the top of each tube on the outside and spin at 23000 x g for 01:00:00 at 4 °C. 1h

1.4 After spinning, remove 300 µL of supernatant and briefly vortex the ~ 200 µL pellet.

> **Note**
>
> Preferably remove ~ 280 µL to ensure you have at least 200 µL of pellet remaining. Pipette from the opposite side of the mark on the top of the tube.

1.5 Proceed to extraction room

Note

Prior to extraction, make 400 µL aliquots of lysis buffer and remove magnetic silica from fridge and equilibrate to room temperature.

1.6 Pipette mix the 200 µL pellet and transfer to 400 µL of lysis buffer.

1.7 Vortex briefly and incubate at room temperature for 00:20:00. 20m

1.8 While the sample is lysing, label disposable NucliSENS easyMAG consumables according to sample list.

1.9 After incubation, transfer lysate (~ 600 µL) into respective wells of NucliSENS easyMAG consumables.

1.10 Vortex magnetic silica briefly and add 50 µL to numbered NucliSENS easyMAG consumables.

1.11 Using a BioHit multichannel pipette set at P3, mix sample solution with magnetic silica by pipetting up and down, avoiding bubbles. Remove excess bubbles if necessary.

1.12 Load NucliSENS easyMAG consumables on to the NucliSENS easyMAG instrument and scan barcodes on consumables.

1.13 Program NucliSENS easyMAG instrument and select 25 µL elution volume.

1.14 Start extraction run.

> **Note**
>
> The run will take ~ 00:35:00

1.15 After extraction is complete, transfer eluent into appropriately labelled 1.5 mL microcentrifuge tubes.

1.16 Discard NucliSENSeasyMAG consumables appropriately as biohazard waste.

1.17 Proceed to perform PCR amplification or store RNA at -80 °C until use.

PCR MASTER MIX

2 Preparation of one-step reverse transcription and second-round PCR master mix

2.1 Proceed to PCR master mix room (i.e. clean room).

2.2 All reagents except the enzymes (Superscript IV and Platinum *Taq* DNA Polymerase) must be thawed and vortexed for 00:00:05 to 00:00:10 15s

2.3 Label reverse transcription and second-round PCR tubes 0.2 mL with sample numbers, accordingly.

2.4 Label two 1.5 mL microcentrifuge tubes as follows; Tube 1: for RT one-step PCR master mix, and Tube 2: for second-round PCR master mix.

> Note
>
> Prepare each master mix for the number of specimens to be amplified, including a negative control, and positive control.

> Note
>
> Add reagents in order listed in Table 1, and make two extra volumes to account for pipetting errors.

2.5

Prepare RT-PCR master mix as shown in Table 1, using Superscript IV one-step PCR reagents.

	A	B	C
	Reagents	**Volume per reaction (µl)**	**Concentration per reaction**
	2X Reaction RT-PCR master mix	12.5	1X
	Nuclease-free Water	2.25	-
	PANA2AF (5µM)	2.5	0.5µM
	PANA3AR (5µM)	2.5	0.5µM
	SSIV/ Platinum SuperFi DNA polymerase (2X)	0.25	0.02X

	A	B	C
	Total volume	**20**	-

Table 1. One-step reverse transcription PCR master mix.

Note

Details of primers for one-step RT-PCR master mix are shown in Table 2.

	A	B	C
	Primer name (Direction)	Primer sequence	**HXB2**
	PANA2AF (Forward)	GAGGCAATGAGCCAARCAAACA	1882 - 1903
	PANA3AR (Reverse)	TTCCAGGGCTCTAGKTTAGG	5846 - 5865

Table 2. Details of primers used in one-step reverse transcription PCR master mix.

Note

Add reagents in order listed in Table 3, and make two extra volumes to account for pipetting errors.

2.6

Prepare master mix for the second-round PCR as shown in Table 3, using Platinum *Taq* PCR reagents.

	A	B	C
	Second-round PCR **Mastermix**		
	Reagent	**Volume per reaction (µl)**	**Concentration per reaction**
	Nuclease-free Water	18.4	-

	A	B	C
	10X PCR Buffer	2.5	1X
	MgCl2 (50mM)	1.0	2mM
	dNTP (10mM)	0.5	0.2mM
	Pro1 (5µM)	0.25	0.05µM
	5066R (5µM)	0.25	0.05µM
	Platinum *Taq* DNA Polymerase	0.1	-
	Total volume	**23**	-

Table 3. Second-round PCR master mix.

Details of primers for second-round PCR master mix are shown in Table 4.

	A	B	C
	Primer name (Direction)	Primer sequences	**HXB2**
	Pro1 (Forward)	TAGAGCCAACAGCCCCACCA	2147 - 2166
	5066R (Reverse)	ATCATCACCTGCCATCTGTTTTC CAT	5041 - 5066

Table 4. Details of primers used in second-round PCR.

2.7 Mix one-step RT-PCR master mix by pipetting up and down (or by gently tapping the tube), and transfer 20 µL to the respective 0.2 µL PCR tubes.

2.8 Mix second-round PCR master mix by pipetting up and down (or by gently tapping the tube), and transfer 23 µL to the respective 0.2 µL PCR tubes.

2.9 Take aliquoted one-step RT-PCR master mix into the extraction laboratory (or dead air space cabinet in general laboratory).

Note

Store second-round PCR master mix at -20 °C until use.

ADDING RNA

3 Adding RNA to one-step RT-PCR master mix.

3.1 Proceed to extraction laboratory, or dead air space cabinet.

3.2 Thaw RNA, gently tap tubes, and briefly spin.

3.3 Add 5 µL RNA to respective tubes containing master mix.

Note

Add 5 µL of nuclease free water to negative control. Total reaction volume is 25 µL.

3.4 Proceed to the PCR amplification room.

ONE-STEP RT-PCR

4 RT-PCR amplification process.

4.1 Gently tap PCR tubes to mix and centrifuge briefly.

4.2 Switch on thermal cycler and wait for the instrument to initialize.

4.3 Place tubes in thermal cycler and run the conditions shown in Table 5.

	A	B	C	D
	Thermocycling conditions	Temperature (degrees celcius)	**Time**	**Cycle(s)**
	cDNA synthesis	50	10 minutes	1
	Pre-denaturation	98	2 minutes	1
	Denaturation	98	10 seconds	40
	Annealing	56	20 seconds	
	Extension	72	2 minutes	
	Final extension	72	10 minutes	
	Hold	4	∞	Hold

Table 5. One-step RT-PCR amplification conditions.

Note

PCR run takes ~ 02:35:00

4.4 After PCR is complete, remove PCR tubes from thermal cycler and proceed to second-round PCR amplification, or store at -20 °C.

SECOND-ROUND PCR

5 Second-round PCR amplification.

5.1 In PCR amplification laboratory, remove second-round PCR master mix from -20 °C.

5.2 Gently mix one-step RT-PCR amplicons by pipetting up and down, and add 2 µL to the respective second-round PCR master mix tubes.

> Note
>
> Total reaction volume is 25 µL.

5.3 Gently tap PCR tubes and centrifuge briefly.

5.4 Place the PCR tubes in thermal cycler and run the conditions shown in Table 6.

	A	B	C	D
	Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
	Pre-denaturation	94	2 minutes	1
	Denaturation	95	10 seconds	40
	Annealing	56	20 seconds	
	Extension	72	2 minutes	
	Final extension	72	10 minutes	1

	A	B	C	D
	Hold	4	∞	Hold

Table 6. Second-round PCR amplification conditions.

Note

PCR run takes ~ 02:15:00 .

5.5 After PCR is complete, remove PCR tubes from thermal cycler and proceed to run gel electrophoresis, or store second-round amplicons at -20 °C .

DETECTION OF AMPLICONS

6 Gel electrophoresis on agarose gel.

6.1 Assemble gel casting apparatus by securing gel casting gates on both sides of the casting tray. Place gel comb in upper slot of the casting tray.

6.2 Prepare 1X TBE by adding 1 part 10X TBE buffer to 9 parts distilled water. For example to make 1 L of 1X TBE, mix 100 mL of 10X TBE with 900 mL of distilled water.

6.3 Prepare 1% agarose gel by adding two 0.5 g agarose tablets to 100 mL of 1X TBE in a Schott bottle.

Note

Break agarose tablets in half before adding to 1X TBE, and wait briefly for tablets to dissolve before heating in microwave.

6.4 Heat in microwave for approximately 00:02:00, swirling occasionally to ensure agarose tablets have dissolved completely. 2m

Note

Take care that the gel-mix does not boil over.

6.5 Gently remove from microwave taking care not to spill over and allow cooling at room temperature, or by running the base of the flask under cold water.

Note

The gel is cool enough when you are able to comfortably touch the gel bottle for 00:00:03 to 00:00:05.

6.6 Add 10 µL of SYBR safe gel stain (10,000X concentrate) to 100 mL gel.

Note

Add 1 µL of SYBR safe gel stain (10,000X concentrate) for every 10 mL) of agarose gel mix.

6.7 Allow agarose gel to set (~ 00:30:00). 30m

6.8 Once set, carefully remove gel comb and the casting gates.

6.9 Place agarose gel in the electrophoresis tank with wells closest to the anode (i.e. negative electrode (black) position on top) and immerse in 1X TBE buffer.

6.10 Add 1 µL of gel loading dye to a piece of parafilm paper for each amplicon to be loaded on the gel.

6.11 Mix with 5 µL of each amplicon and load into respective wells of the agarose gel.

> **Note**
>
> Preferably, start loading amplicons from the second well, taking note of the order by which the amplicons are loaded.

6.12 Mix 3 µL of gel loading dye with 3 µL of DNA ladder, and load into the first well of the agarose gel.

6.13 Close the lid of the electrophoresis tank ensuring that the negative electrode (black) is positioned close to the agarose gel wells.

6.14 Switch PowerPac on and set voltage at 120V.

6.15 Run the gel for at least 00:45:00 . 45m

Note

Check for bubbles at negative electrode (black) to ensure there is current running through the tank.

6.16 After running gel, remove it from electrophoresis tank and visualize on BioMetra UV detection unit.

6.17 PCR amplicons should be at a size of approximately 2.9 kilobases (kb) as measured against the DNA ladder in well position 1.

Note

For a valid PCR run, the positive control should have a band at approximately 2.9kb, and the negative control should not have a band.

6.18 Proceed to PCR product purification or store amplicons at -20 °C until use.

PCR PRODUCT PURIFICATION

7 PCR product purification using ExoSAP-IT PCR production purification

7.1 For samples with successful amplification, label PCR tubes required for purification.

7.2 Tap amplicon tubes gently to mix and briefly spin to bring the contents to bottom of tube.

Note

If frozen, first thaw the amplicons to room temperature.

7.3 Remove ExoSAP-IT Express reagent from -20 °C freezer.

7.4 Gently tap ExoSAP-IT Express reagent to mix and briefly spin to bring contents to bottom of tube.

7.5 Mix 10 µL of PCR amplicon with 4 µL of ExoSAP-IT Express reagent for a combined 14 µL reaction volume.

7.6 Mix thoroughly by tapping the tube and quick spin to bring contents to the bottom of the tube.

7.7 Place the tubes in thermal cycler and run the conditions shown in Table 7.

	A	B	C	D
	Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
	Cleanup	37	4 minutes	1
	Enzyme inactivation	80	1 minute	1
	Hold	4	∞	Hold

Table 7. PCR purification conditions.

7.8 After ~ 00:05:00 on thermal cycler, purified PCR products are ready for DNA sequencing. 5m

Note

Purified PCR products may be stored at -20 °C until use.

CYCLE SEQUENCING REACTION AND PURIFICATION

8 BigDye cycle sequencing.

8.1 The following procedure is used to set up the sequencing reaction mix using BigDye Terminator v3.1 cycle sequencing kit (LifeTechnologies) with 8 primers per sample.

8.2 Thaw all reagents at room temperature.

8.3 Label eight 1.5 mL tubes, one for each primer mix.

8.4 Vortex sequencing buffer and primers and briefly spin to bring contents to the bottom of the tube.

8.5 Mix BigDye reagent by tapping tube and briefly spin to bring contents to bottom of tube.

Note

Add reagents in order listed in Table 8, and make two extra volumes to account for pipetting errors.

8.6 Prepare master mix for each primer as shown in Table 8.

	A	B	C
	Reagent	**Volume (µl)/ reaction**	Concentration per reaction
	Nuclease-free Water	6.1	-
	5X Sequencing buffer	2	1X
	Primer (3.2µM)	0.5	0.2µM
	BigDye Sequencing mix	0.4	-
	Total	**9.0**	

Table 8. BigDye cycle sequencing reaction mix.

Note

Details of sequencing primers are shown in Table 9.

	A	B	C	D
	Primer (Direction)	**Primer sequence**	HXB2	**Gene**
	RTC1F (Forward)	ACCTACACCTGTCAACATAATTG	2486 - 2508	PR and RT
	RTC2R (Reverse)	TGTCAATGGCCATTGTTTAACCTTTGG	2630 - 2604	PR and RT
	RTC3F (Forward)	CACCAGGGATTAGATATCAATATAATGTGC	2965 - 2994	PR and RT
	RTC4R (Reverse)	CTAAATCAGATCCTACATACAAGTCATCC	3101 - 3129	PR and RT
	KVL076 (Forward)	GCACAYAAAGGRATTGGAGGAAATGAAC	4161 - 4188	IN
	KVL082 (Forward)	GGVATTCCCTACAATCCCCAAAG	4647 - 4669	IN

	A	B	C	D
	KVL083 (Reverse)	GAATACTGCCATTTGTACTGCTG	4750 - 4772	IN
	PAN2R (Reverse)	CTGCCATCTGTTTTCCATAYTC	5037 - 5058	IN

Table 9. Details of sequencing primers.

8.7 Label 96-well plate for the cycle sequencing reaction PCR.

8.8 Dispense 9 µL of each primer mix into the respective wells of a 96-well plate.

8.9 Add 1 µL purified amplicons to the respective wells.

Note

It is recommended to add the eight primers across the plate (e.g. primer RTC1F in wells A1 - A12), with samples added in plate columns (e.g. sample 1 in wells A1 - H1).

8.10 Cover plate with aluminium plate seal, tap plate gently to mix, and spin plate briefly.

8.11 Remove aluminium plate seal and cover plate with PCR septa mat.

8.12 Place on thermal cycler and run the conditions shown in Table 10.

	A	B	C	D

	A	B	C	D
	Thermocycling conditions	**Temperature (degrees celcius)**	**Time**	**Cycle(s)**
	Pre-denaturation	96	1 minutes	1
	Denaturation	96	10 seconds	35
	Annealing	50	5 seconds	
	Extension	60	4 minutes	
	Hold	4	∞	

Table 10. Cycle sequencing reaction conditions.

8.13 After the cycle sequencing reaction run is complete, proceed with BigDye sequencing reaction purification, or store the plate covered in foil at 4 °C for up to 1 week, or at -20 °C for storage longer than 1 week.

8.14 Remove SAM and XTerminator reagents from fridge (stored at 4 °C) and equilibrate to room temperature prior to preparing SAM/BigDye XTerminator mix.

Note

The BigDye XTerminator Purification kit (Life Technologies) is used to purify sequencing reaction products. The kit consists of 2 reagents; XTerminator solution and SAM solution.

8.15 Following BigDye cycle sequencing reaction, centrifuge 96-well reaction plate at 4000 rpm for 00:01:00. 1m

8.16 Thoroughly vortex XTerminator solution for at least 00:00:30. 30s

8.17 Prepare the SAM/BigDye XTerminator bead working solution in a 50 mL Falcon tube, by adding reagent volumes shown in Table 11.

	A	B
	Component	**Volume per 10µL reaction**
	SAM solution	45µL
	BigDye XTerminator solution	10µL
	Total volume	**55µL**

Note

Preferably use wide-orifice tips when pipetting BigDye XTerminator solution.

8.18 Mix the SAM/BigDye XTerminator bead working solution thoroughly by vortexing and dispense in a reagent reservoir.

8.19 Remove PCR septa mat from 96-well reaction plate and add 55 µL of SAM/BigDye XTerminator bead working solution to each well.

Note

Preferably use a multi-channel pipette to add reagent mix, changing tips each time.

8.20 Cover plate with aluminium plate seal.

8.21 Vortex plate on a plate shaker at 1800 rpm for 00:30:00 . 30m

8.22 After vortexing, centrifuge plate at 4000 rpm for 00:02:00 . 2m

Note

If the reaction plates are not run immediately, you can store them under the following conditions:

- Room temperature (i.e. 20 °C - 25 °C): Store plates sealed with adhesive film or septa for up to 2 days.
- Refrigerated storage (4 °C): Store plates sealed with adhesive film for up to 10 days.
- Frozen storage (-20 °C): Store plates sealed with adhesive film for up to 10 days.

CAPILLARY ELECTROPHORESIS

9 Capillary electrophoresis on 3730 Genetic Analyzer

9.1 Setup plate run on Genetic Analyzer (Applied Biosystems), according to cycle sequencing reaction plate layout.

9.2 Load plate onto Genetic Analyzer (Applied Biosystems) and start capillary electrophoresis run.

9.3 At the end of the run, remove the plate from the Genetic Analyzer and dispose in biohazard waste.

9.4 Login to SeqA6 software on a computer connected to the Genetic Analyzer and run primary analysis.

9.5 Save the analyzed data files, and copy the ab1 sequence files to USB disk.

SEQUENCE ANALYSIS

10 Sequence editing and drug resistance interpretation.

10.1 Upload ab1 sequence files from USB key into Geneious software (Biomatters Ltd, New Zealand).

10.2 Trim and edit sequences for good quality reads.

Note

If you are in the correct reading frame, the beginning of protease gene should start with the amino acids PQITLW, the beginning of reverse transcriptase gene should start with PISPIE, and the beginning of integrase gene should start with FLDGID.

10.3 Align all sequence files for each sample to an annotated HIV reference sequence, and edit sequences where necessary. This entails identifying any ambiguities, and verifying positions with mixed bases by inspecting quality (symmetry, height, and background) of the base calls.

10.4 Align all consensus sequences and perform phylogenetic tree reconstruction to assess sequence similarity.

Note

If any two sequences have >97% similarity, all the stages of the protocol should be reviewed, starting with sequence analysis and going back to RNA extraction, to ensure that there was no sample mix up (i.e. sample switching, mislabeling), or contamination.

10.5

Export consensus sequences in FASTA format and assess HIV drug resistance mutations using the Stanford University HIV drug resistance database

(https://hivdb.stanford.edu/hivdb/by-sequences/).