# Chapter 4

**Development of Seq2Res: A computational Drug Resistance Testing Pipeline for the Analysis and Management of HIV-1 Roche/454 Sequence Data**

## 4.1 Introduction

UNAIDS estimates that approximately 34 million individuals - including 2.6 million newly infected - were living with HIV and as many as 1.6 million died of HIV-related illnesses in the year 2011 alone (UNAIDS, 2012). Sub-Saharan African region has the highest prevalence of HIV infections (Asamoah-Odei et al., 2004; Hamers et al., 2011a). Antiretroviral therapies (ARTs) are rolled out (Blower et al., 2005; Herbst et al., 2009; Nattrass, 2006; Stringer et al., 2006) in the region to suppress viral replication, resulting in a lower viral load (Autran et al., 1997; Li et al., 1998; Mocroft et al., 2010) and thereby extending the life expectancy of HIV positive individuals (Fang et al., 2007; Harrison et al., 2010; Mills et al., 2011). By the end of 2011, 8 million people from low and middle-income countries were receiving the life saving drugs (20 times more than in 2003) (UNAIDS, 2012; WHO, 2011).

HIV produce new viruses in the order of 1010 per replication cycle and mutates in the order of 10-5 per nucleotide per replication cycle (Ho et al., 1995; Mansky, 1996a, b, 1998; Mansky and Temin, 1995). The high mutation rate is due to the error prone reverse transcriptase enzyme that transcribes viral RNA to cDNA during the replication (Bebenek et al., 1989; Berkhout et al., 2001; Preston et al., 1988; Roberts et al., 1988). The accumulation of random mutations in HIV leads to development of drug resistance (Johnson et al., 2008).

With scale up of antiretroviral (ARV) drugs there are growing evidences suggesting that drug resistant HIV virus exist as minor variants in the population under therapy (Aghokeng et al., 2011; Dudley et al., 2012; Gupta et al., 2012; Lataillade et al., 2010; Li et al., 2011; Little et al., 2002; Simen et al., 2009a; Yebra et al., 2011). This observation is also supported by several research studies on population-level surveillance of HIV drug resistant variants (Dudley et al., 2012; Hamers et al., 2012; Hamers et al., 2011a; Hamers et al., 2011b; Lataillade et al., 2010; Li et al., 2011; Little et al., 2002; Simen et al., 2009a). A clinical reason for the increase in drug resistant viral variants is an interruption to drug adherence during therapy (Bangsberg et al., 2003; Golin et al., 2002; Low-Beer et al., 2000) and transmission of resistant viral variant to HIV negative individuals (Jakobsen et al., 2010; Supervie et al., 2010; Wittkop et al., 2011; Yerly et al., 1999). The rising prevalence of low-level drug resistant variants narrows treatment options (Hamers et al., 2012; Hamers et al., 2011a; Hamers et al., 2011b). Thus, ARV treatment, management and surveillance of drug resistant HIV variants (Bennett et al., 2009; Bennett et al., 2008) is essential for prolonging the usage of, and optimizing the outcome from, a particular drugs cocktail (Adetunji et al., 2012). Therefore, the World Health Organization (WHO) guideline ([http://www.**who**.int/**hiv**/drugresistance/](http://www.who.int/hiv/drugresistance/)) recommends pretreatment HIV drug resistance test, but this has only been possible in rich and developed countries (Aberg et al., 2009; Clumeck et al., 2008; Gazzard et al., 2008; Kaplan et al., 2009; Masur and Kaplan, 2009; Perfect et al., 2010).

Both individual and population-level screening of resistant HIV variants must be done routinely for tracking of resistant viruses and to prevent the exhaustion of ART treatment options. However, due to the cost factor, regular pretreatment resistance testing, in low and middle-income countries, was not possible until the advent of high throughput Sequencing (HTS). HTS is a robust, reliable and affordable way out for exploring clinically relevant low abundance (< 1%) resistant HIV variants (Dudley et al., 2012; Ji et al., 2012; Tsibris et al., 2009; Wang et al., 2007). The huge volume of sequence data from HTS is a challenge for data analysis and management. Further, there have been no publications of web-based tool for routine drug resistance test and clinical management of HIV infected individuals and drug resistant mutations using HTS method. This chapter describes the development and application of such a computational pipeline tool to facilitate low cost HIV drug resistance test using HTS technology. A front-end web API (available at https://hiv.sanbi.ac.za/tools/seq2res) is also developed and maintained for anyone with least computational expertise.

## 4.2 Methods and Materials

### 4.2.1 HTS raw sequence reads

A set of forward and reverse primers that bind at targeted genome regions in the HIV genome and is PCR amplified to produce millions of copies of the region. Each copy of the region is then sequenced using HTS method. In an instance, when multiple samples need to be sequenced, the forward and reverse primers set is first attached to the 3’ end of a unique sample specific sequence, which is now described as Multiplex Identifier (MID) (**Figure 4.1**).

### 4.2.2 Development and processing of data using Seq2Res

A computational pipeline named Seq2Res was developed for HIV drug resistance testing of sequences generated using Roche/454 High Throughput Sequencing (HTS)

Platform. Users need to input raw sequence data file, the primers used in resequencing a genomic region and if, present, the sample specific sequence information or multiplex identifier (MID). The primer file is a five column tab-delimited file containing the name of amplicon in the first column, forward and reverse primers in the second and third column and amplicon start and end positions relative to the standard HIV pol reference sequence in the fourth and fifth columns respectively (**Figure 4.2 A**). The MID file contains tab delimited two columns of data of which the first column contains the Roche/454 standard MID name or the actual nucleotide sequence of the MID used and the second column contains the sample name or the sample name to which the MID is applied (**Figure 4.2 B**). The end-users also have an option to choose following required preprocessing input parameters (**Figure 4.3 Step 1**):

1. Number of nucleotide mismatches between user supplied MID and MID in sequence read, which is described as MID tolerance
2. Number of nucleotide mismatches between user supplied primer and the primer in the sequence read, which is described as primer tolerance
3. Quality control parameters for raw data processing.

The details of each Seq2Res processing step is given below:

1. **File format pre-processing**

Raw sequence data can be uploaded in one of the three different sequence file formats to the pipeline: a Standard Flowgram Format (SFF) file generated by Roche/454 HTS platform, a FASTQ file, or a combination of FASTA and QUAL files. Depending on the input file, Seq2Res may need pre-processing conversion of the file (**Figure 4.3 ste 2**). Since the subsequent steps in the Seq2Res pipeline work only with FASTQ files, the other formats are converted to FASTQ format during the pre-processing. An SFF file is converted to a FASTQ file with a tool called sff\_extractor (<http://bioinf.comav.upv.es/sff_extract/index.htm>); a FASTA file with a paired quality scores file is merged to a FASTQ file.

1. **Preprocessing of MID and Primer files**

Seq2Res reads the tab delimited MID sequences and the sample names from the MID file and stores the list of paired sample name and its corresponding MID sequence. Seq2Res also reads the tab-delimited primer file containing the name of amplicons, primers (forward and reverse primers), the start and end nucleotide positions of amplicons (**Figure 4.3 step 3**). Seq2Res stores the amplicon names associating with its primers and start-end positions. From the start and end nucleotide positions, Seq2Res processes to find the start and end codon positions in the standard HIV *pol* reference sequence. On the basis of the start and end codon positions of each amplicon, Seq2Res again processes to find the first and the last DRM codon positions of the amplicons in the HIV *pol* reference sequence. The amplicon size from the first and the last DRM codon positions is called the optimal coverage amplicon. Seq2Res now defines a sequence that has the first DRM position and ends at the last DRM position or beyond as the optimal coverage full-length sequence.

1. **Demultiplexing**

Seq2Res utilizes Biopython ([www.biopython.org](http://www.biopython.org)) (Cock et al., 2009) packages to read sequences from FASTQ file. Seq2Res searches for MID as the first step of demultiplex. MID is located at 5’ end of a sequence read. A subsequence of length equal to the length of MID is obtained from 5’ end of the sequence read. The subsequence is then pair-wise global aligned with every MIDs in the input list of MIDs. The MID with the number of mismatches less or equal to MID tolerance in the alignment (Default MID tolerance 2) is added at the sequence ID and the MID subsequence is removed from the sequence read. If none of the MIDs have MID tolerance less or equal to the threshold, the sequence read is discarded. Next, the primer is searched in the sequence read in the similar way as MID. Again, a subsequence of the length equal to a primer length is obtained from 5’ end of the sequence read. The subsequence is then pair-wise global aligned with every primer in the input list of forward and reverse primers. The primer either forward or reverse with the number of mismatches less or equal to user threshold or primer tolerance (Default primer tolerance 3) is selected and tagged at the sequence ID along with the strand forward (+) or strand reverse (-). If the sequence reads are generated using Primer ID technology, Seq2Res searches primer, MID and Primer ID as in PIDA algorithm.

1. **Advanced Sequence Reads Quality Control**

The non-discarded sequences are quality trimmed in the next step. During sequencing, Roche/454 HTS calls a base with a certain probability, and this score for each base is saved in the quality file. The scores range from minimum of zero to a maximum of 40. A score of 10 would mean that the probability of a wrong base call is 1 in 10, 20 means 1 in a 100, and 40 means 1 in a 10000. In general, the quality scores of a base decrease as the sequence length increases. A lower quality score indicates that a base has a lower probability of being correct and including these poor quality bases would compromise the data analysis. Seq2Res uses QTrim (<https://hiv.sanbi.ac.za/tools/qtrim>) – an in-house designed quality-trimming tool for removing these lower quality bases. Seq2Res allows a flexibility to control all the features of QTrim.

1. **Sequence grouping by Primer and MID**

The ‘QTrim trimmed’ sequences are grouped by sample/MID, and amplicon names/primer. Seq2Res gets the MID and primer information for the sequence reads from their sequence ID.

1. **Generation of consensus sequence for sequence with Primer ID**

For Primer ID (Jabara et al., 2011) data, the sequences with the same Primer ID are further grouped together. MAFFT (Katoh et al., 2005; Katoh et al., 2002; Katoh and Toh, 2008, 2010) is used for the multiple sequence alignment of same Primer ID sequences to generate a consensus sequence that takes into account the most occurring base in a position. For a tie between two or more bases at a position, an ambiguous base representing the bases is added in the consensus sequence. Consensus sequences from the same genomic amplicon and sample are grouped together.

1. **Reference Mapping**

Sequences are aligned with a standard reference sequence to identify any mutations in the nucleotide sequences. Sequences grouped by same genomic amplicon and sample is reference mapped using mapping tool called RAMICS (Rapid Amplicon Mapping In Codon Space) (submitted for publication). Seq2Res uses RAMICS to map the genomic amplicon sequences from the first to the last DRM codon position. RAMICS discards poorly aligned sequences and outputs a FASTA like FASTM file containing a sequence ID and comma-delimited codon positions that are with respect to the reference sequence followed by an amino acid at the positions in the mapped sequence **(Figure 4.4)**. If a mapped sequence covers the first and the last DRM codon position of its amplicon, RAMICS adds information “Y” (for “yes”) to indicate that the mapped sequence is full length or “N” (for “No”) for not full length at the end of the sequence ID.

1. **Codon position translation**

Seq2Res translates codon positions with respect to the reference sequence (the HIV *pol*) in FASTM file into codon positions with respect to genomic amplicon region (protease or reverse transcriptase or integrase) with the amino acids in the codon positions unchanged (**Figure 4.5**). For example, the codon positions 57 and 156 in HIV *pol* sequence are translated to protease codon position 1 and reverse transcriptase codon position 1 respectively. From the list of translated codon positions, only the positions that are in the list of standard drug resistant mutation codon positions (**Table 4.1**) are selected for a sequence read. Out of the translated codon positions of a sequence, a codon position with unknown amino acid is removed from further analysis and the rest codon positions of the sequence are retained. Translated codon positions with their amino acids from every sequence from all amplicons of a specific sample, are saved together in a file for HIV drug resistance testing of the sample.

1. **Resistance Testing**

The file containing all the translated drug resistant mutation codon positions with amino acids from all amplicons of a sample is then submitted to a locally installed Sierra genotypic resistance interpretation algorithms (Liu and Shafer, 2006) that use sequence reads codon positions to query Stanford HIV database (Rhee et al., 2003; Shafer, 2006) for resistance. For the submitted file containing codon positions and its amino acids, the Sierra algorithm outputs the result of the query in a XML file containing the drug resistant mutation codon positions with their amino acids and their drug resistance level – Resistant (R), Intermediate (I) or Susceptible (S) - for each codon position with amino acids against all clinical antiretroviral drugs in three drug classes - Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleotide Reverse Transcriptase Inhibitors (NNRTIs) and Protease Inhibitors (PI).

1. **Resistance report presentation**

Seq2Res extracts the resistance level for every DRMs in the sequences from the XML file, and tabulates it in a tab-delimited file. Each row in the tabulated file contains user and sequence information in the first ten columns. The order of the information in the first ten columns are USERID, JOBID, input file name, sample name, the MID of the sample, a sequence read ID, number of the sequences with that sequence read ID, the amplicon name of the sequence, strand orientation (forward or reverse) of the sequence and the length of the sequence. From column 11, a resistance level R, I or S appears at odd columns and DRMs of the sequence that are responsible for the resistance level for a drug in even columns in the order of specific sequence of drugs (**Table 4.2**). For example, a resistance level “R” may appear at column 11 and DRMs K65R, K70R, V75I, F77L from a sequence that are resistant (R) to the first drug Abacavir (ABC) may appear in column 12. Similarly in column 13, resistance level “I” may appear while in column 14, the DRM F77L may appear that is intermediate resistance to the second drug Didanosine (DDI). If the DRMs that give resistance to a particular drug are not present in the sequence, a dash (” -“ ) appears in the columns for resistance level and DRMs for that drug.

From the table, the prevalence of sequence reads that are highly resistant (R), intermediate resistant (I) or susceptible (S) to a particular antiretroviral drug are calculated. The prevalence of resistant and intermediate sequences for an antiretroviral drug are compared with the user defined cutoff prevalence and the antiretroviral drug is classified as resistance or intermediate resistance or susceptible to a drug using the following conditions in order:

1. If the prevalence of resistant sequences to a drug is greater than user defined cutoff, the drug is classified as Resistant.
2. If the prevalence of resistant sequences and intermediate resistant sequences to a drug is collectively greater or equal to user-defined cutoff or the prevalence of intermediate resistance to a drug is greater than user defined cutoff, the drug is classified as intermediate resistant.
3. If not both of the above, then the drug is classified as susceptible.

The antiretroviral drugs and their classification as resistant, intermediate or susceptible are tabulated as high throughput drug resistant report. Each classified drug is color-coded in the report as – Red for highly resistant, Orange for intermediate resistant and green for susceptible. The frequency of drug resistant mutations (DRMs) is also calculated from RAMICS output FASTM file and generates an “ultra deep prevalence plot” showing the prevalence of each DRM and a horizontal red line representing the cutoff prevalence.

1. **Consensus like report generation**

In a consensus like analysis, the DRMs with prevalence greater or equal to the defined cutoff are selected in each amplicon of a sample. If there are overlaps between the amplicons and DRMs occur in the overlap region, the DRM with higher prevalence of resisatance is selected. Multiple DRMs at the same codon position but with different mutations are also selected. The selected DRMs are submitted to local Sierra genotypic resistance interpretation algorithms (Liu and Shafer, 2006) as a single sequence DRMs for resistance test. The algorithm generates a XML file from which a drug resistant report for the consensus like sequence DRMs is generated that shows the drug susceptibility with color code similar to the high throughput drug resistant report for all antiretroviral drugs.

### 4.2.3 Test Data for comparison of web sierra and Seq2Res

Two test data sets - “Large data set” and “very large data set” were downloaded from Stanford Database website (<http://hivdb.stanford.edu/DR/asi/releaseNotes/index.html#sampledata>). Both the data sets were generated using population based Sanger Sequencing method covering the entire protease and reverse transcriptase region of HIV. There were 2055 sequences in “large data set” and 5838 sequences in “very large data set”. Both the data sets were submitted to web Sierra (Liu and Shafer, 2006) and the results displayed in spreadsheet format was saved in a file. In order to run the two test data in Seq2Res, the sequences in both the test data were fragmented into three subsequences per sequence with an overlap of 100 nucleotides between the adjacent subsequences. The first subsequence covers the entire HIV protease gene and so it is called as fragment PR. The second subsequence covers the first half of HIV reverse transcriptase gene, so it is called RT1 fragment and the third subsequence covers the second half of HIV reverse transcriptase gene, so it is called RT2 fragment. The fragments PR, RT1 and RT2 were then mapped to a standard HIV *pol* sequence using Seq2Res mapping tool – RAMICS. RAMICS outputs the amino acids at each codon positions of the sequences after mapping which were then submitted to local sierra. The local sierra result for fragments RT1 and RT2 were merged together as they come from same reverse transcriptase gene. The results from web Sierra and RAMICS mapping and local sierra were compared.

### 4.2.4 Test Data for simulation

Five HIV sequences, each from different samples, generated using population based Sanger Sequencing method and covered the entire protease and reverse transcriptase genes were selected from a “very large data set” available at Stanford Database website (<http://hivdb.stanford.edu/DR/asi/releaseNotes/index.html#sampledata>). The sequences were chosen such that each sequence has at least K65R, K103N mutations and any other reverse transcriptase mutations along with one or more protease resistant mutations. Ambiguous bases, if any, at a DRM codon position in the sequences were replaced with a base that give mutant amino acids while at non DRM codon position was replaced with any representing base. A label “Resistant” was added to the sequence ID (as they have DRMs) with the sample name for the sequences as obtained from Stanford Database and saved as FASTA file. A copy of each sequence was created and all the DRMs **were changed to wild type** manually. A label “Susceptible” was added to the sequence ID (as no DRMs) with the same sample name as its resistant sequence and saved in the same FASTA file. The FASTA file contained ten sequences in total – five resistant and five susceptible sequences. The FASTA file was then submitted to web sierra.

### 4.2.5 Simulation of test data

Further, each sequence in the FASTA file (containing 5 resistant and 5 sensitive sequences) was then fragmented into three subsequences, each of length 600 nucleotides, such that there was large overlap between the adjacent subsequences. This generated a new FASTA file containing 30 fragmented sequences (three from each sequence); the name Protease (PR) for first fragment, Reverse Transcriptase 1 (RT1) for second fragment and Reverse Transcriptase 2 (RT2) for third fragment was added to the sequence ID.

### 4.2.5.1 Running Simulator

ART is a next generation sequence data simulator tool (Huang et al., 2012). ART can create a sequence profile from Roche/454 real sequence data and use the profile to generate Roche/454 sequence data with real sequencing errors from a single sequence. The fragment PR, RT1 and RT2 FASTA sequence file was submitted to the ART tool to simulate 20,000 Roche/454 sequence reads for each fragment, now called amplicon sequences, in FASTQ format.

### 4.2.5.2Generation of different known prevalence of DRM data

Using the first 30 nucleotides of each amplicon sequence as primer information, simulated sequences were pooled together using in-house script. Approximately 10,000 simulated sequences, all in forward orientation, were pooled from a set of 20,000 simulated reads generated for both “Resistant” and “Susceptible” PR, RT1 or RT2 amplicons. The resistant and susceptible pooled forward sequences from same PR or RT1 or RT2 were mixed in a proportion such that the percentage of resistant sequences are 0.1%, 1%, 5%, 10%, 15%, 20% and 50% and the total number of sequences was 10,000 in all mixtures (**Table 4.1**). For example, 500 sequences of resistant PR amplicon and 9500 sequences of susceptible PR amplicon from same sample were mixed to obtain 5% resistant sequences in the mixture. The mixtures of “Resistant” and “Susceptible” sequences were saved in separate FASTQ files. For each amplicon, seven FASTQ files each with different prevalence of resistant sequences were created. In total there were 105 (7 prevalences x 3 amplicon x 5 samples) FASTQ files. The FASTQ files were submitted to Seq2Res through web API (<http://hiv.sanbi.ac.za/tools#/seq2res>).

### 4.2.6 Computational Infrastructures

Seq2Res executes in the computational infrastructure at the South African National Bioinformatics Institute (SANBI). Currently, there are two Blade Servers (PowerEdge M610x) at SANBI, each comprising 12 core processors (Intel(R) Xeon(R) CPU X5675 @ 3.07GHz), 32-gigabyte memory and a graphical processing unit (GPUs) (NVIDIA®Tesla™ M2090).

### 4.3 Results

### 4.3.1 Comparison of Mutation call and Resistance Level Call for the Mutations from Web Sierra and Seq2Res

For the submitted “large data set” and “very large data sets”, web Sierra reported mutated amino acids at the drug resistant codon positions and the resistance level – R, I or S - of those drug resistant mutated amino acids. Seq2Res mapped the sequences to HIV *pol* reference sequence and reported the amino acids at all the codon positions, which are then translated to codon position in genomic region and then submitted to local sierra, which reported the resistance level of amino acids at drug resistant codon positions.

The comparison of DRMs reported by web Sierra and Seq2Res mapping tool – RAMICS showed that except a single drug resistant mutation in reverse transcriptase codon position 236 in a sequence from “large data set”, there was a 100% congruency in mutation calls. The web Sierra reported amino acid Leucine (236L) while RAMICS reported wild type amino acid Proline (236P) at the discordant codon position.

The alignment of the reference sequence and the sequence containing the discordant codon position was obtained from RAMICS sequence alignment output. We manually reviewed the alignment in an alignment viewer tool. RAMICS showed that there was a deletion of nucleotide ‘T’ in the codon position 234, which result to unknown amino acid at that position (**Figure 4.6 1A**). The alignment at the codon positions after the codon position 234 showed wide type (**Figure 4.6 1B**). On the other hand, bases on the alignment reported by web Sierra, amino acid at codon positions 234 was proline (P) while wild type was leucine (L), at codon position 235 was isoleucine while wild type was histamine (H) and at codon position 236 was leucine while wild type was proline (P). Based on this codon positions information, we removed the deletion manually at codon position 234 from RAMICS alignment. The removal of the deletion showed exactly the same amino acids at codon positions 234, 235 and 236 as reported by web Sierra. Therefore, we concluded that web Sierra alignment had no deletion at codon position 234 (**Figure 4.6 2C**). This resulted the rest of the sequences to go out of reading frame, reporting different amino acids than the wild type at all codon positions (**Figure 4.6 2D)**.

### 4.3.2 Drug Resistant Mutations in the selected test sequences for simulation

Out of the ten test sequences for simulation, the five sequences were confirmed with mutations that are highly resistant to most of the protease inhibitors and the reverse transcriptase inhibitors, whereas the five sequences were confirmed without any mutations (**Table 4.4**) by submitting the sequences to web sierra (<http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput>). Web Sierra reported the drug resistivity to DRMs in five resistance levels - high-level resistance, intermediate resistance, low-level resistance, potential low-level resistance and susceptible – that are converted to three resistance level – Resistance (R), Intermediate (I) and Susceptible (S) - according to Stanford HIVDB algorithm (**Table 4.5**). The five resistant sequences with DRMs were observed to be highly resistant to most of the antiretroviral drugs while five sequences without any DRMs were observed to be sensitive to the antiretroviral drugs (**Table 4.6**).

### 4.3.2.1. Seq2Res analysis of simulated data

**Initial demultiplexing**

All 105 simulated datasets, each containing a total number of 10,000 sequence reads and different proportions of resistant and sensitive sequence reads were analyzed with Seq2Res independently. In Seq2Res, initial demultiplex step involved identifying sequence reads that had no primer and MID tag sequence and discarding them from subsequent analysis.

The simulated datasets had no MID tag sequence and thus, demultiplexing the sequence reads with MID tag sequence was skipped. The information “NOMID” was added to the sequence ID. In the next step, sequence reads were demultiplexed using primer information. There was no any sequence reads discarded in this step and that was because sequence reads in the input file were already selected from a larger simulated dataset generated by high throughput simulating tool ART (Huang et al., 2012) using primer before mixing the resistant and sensitive sequence reads. Thus, 100% of sequence reads were passed through subsequent analysis.

**Quality trimming of simulated datasets**

All the sequence reads in every simulated dataset were quality trimmed using QTrim at mean quality score of 20. Interestingly, not a single sequence read was discarded from all 105 simulated datasets. This showed that high quality simulated sequence reads were simulated from ART (Huang et al., 2012) simulator.

In the subsequent analysis, sequence reads were binned together by amplicon and not by MID as no MID information was provided. There was only one amplicon in each dataset; therefore, all the sequences were binned together resulting in only one dataset in all 105 datasets. The dataset generated by binning was subsequently analyzed.

**Reference mapping of binned dataset and optimal full-length evaluation**

Every sequence reads in the dataset generated by binning were mapped to a standard HIV *pol* reference sequence using a mapping tool – RAMICS. For every mapped sequence reads, RAMICS added information either “Y” for optimal full-length if a sequence read was extended from the first to the last DRM positions of its amplicon or “N” if it was not.

HIV drug resistance testing required that a sequence read covered all DRM position of its amplicon. Therefore, sequence reads that were not optimal full-length were discarded from subsequent analysis. The number of sequence reads that were discarded as non optimal full-length was observed to be correlated for a specific amplicon across the five samples and the seven predefined prevalence levels. The average number of sequence reads discarded in amplicon PR was 180, in amplicon RT1 was 2535 and for RT2 was 396 across the five samples and seven predefined prevalence levels (**Table 4.7**). Surprisingly, the number of sequence reads for sample 56252 in amplicon RT2 was none at all seven predefined prevalence levels while other samples had lost an average of 500 sequence reads for the same amplicon across the seven predefined prevalence levels. Thus, for RT2 amplicon an average number of sequence reads discarded as non optimal full-length reduced to 396. The resulting dataset of optimal full-length sequence reads were passed for subsequent downstream analysis.

**Codon translation and Resistance testing**

RAMICS output FASTA format like FASTM file that reported a sequence ID followed by a list of comma separated codon positions with respect to the reference sequence and amino acids at those positions of every full-length sequence read. The codon positions were translated to the codon positions with respect to the gene and only the codon positions that were in the list of standard drug resistant mutation codon positions for the gene were selected. Subsequently, these selected codon positions of the sequences were saved in a file and analyzed in the subsequent downstream processes.

The file containing the translated and selected drug resistant mutation codon positions with amino acids was passed through multiple genotypic resistance interpretation algorithms in local Sierra (Liu and Shafer, 2006) for HIV drug resistance testing on every codon position of a sequence. Sierra algorithm produced an output XML file containing a resistance level –R, I or S - for codon position in every sequence read.

**Prevalence of known drug resistant mutations**

For every sequence read, the drug resistant mutations of the sequences and their resistance levels were extracted and tabulated in tab-delimited file such that for defined sequence of antiretroviral drugs, the table listed out the resistance level R, I or S and the DRMs of a sequence for each drug. The tab-delimited file was obtained for all 105 input simulated dataset.

Sequence reads in each datasets were derived from a resistant and a sensitive sequence reads by simulation, and therefore, the dataset derived from a sample’s resistant and sensitive sequence must have the DRMs from the resistant sequence as reported by web Sierra (Liu and Shafer, 2006).

Of the 105 datasets, every simulated dataset was analyzed for the prevalence of DRMs that it contained from tab-delimited file. From a tabulated file for every input simulated dataset, out of the total sequences that went through drug resistance testing in local Sierra, the prevalence of sequence reads containing each known mutations for the sample was calculated (**Figure 4.7**).

Each simulated dataset had defined proportion of resistant and sensitive sequences and therefore, the prevalence of known mutations in the dataset would be expected to be similar to the prevalence of resistant sequences in the mixture. Seq2Res analysis of simulated data showed that the observed prevalences of the known mutations in most of the amplicons were so close to the expected prevalence that they were almost equal (**Figure 4.7**).

Analysis of all the observed prevalences across the samples from the same expected prevalence level showed that the mean of the observed prevalence (up to the first decimal value) are equal to the corresponding expected prevalence and the standard deviation is less than 1 except at the expected prevalence of 50% (**Table 4.8**). The high standard deviation at expected prevalence of 50% was due to higher observed prevalence (55.42%) for DRM K103N in amplicon RT2 of sample 2368 (**Figure 4.7 E**) and lower observed prevalence (46.75%) for DRM K65R in amplicon RT1 of sample 56252 (**Figure 4.7 A**) than expected prevalence of 50%.

Further analysis on the dataset of sample 2368’s amplicon RT2 at 50% expected prevalence showed that there were 4440 sequences with DRM K103N and 5057 sequences without DRM K103N in the final analysis result. As sequences with DRM were lower than sequences without DRM, the observed prevalence was lower than expected prevalence.

Similarly, in dataset of sample 56252’s amplicon RT1 at 50% expected prevalence showed that there were 4881 sequences with DRM K65R and 3780 sequences without DRM K65R in the final analysis. Therefore, the observed prevalence was higher than the expected prevalence.

**Effect of simulation error in the observed prevalence**

All 105 simulated datasets were prepared with a fixed proportion of resistant and sensitive simulated sequence, thus, the number of simulated resistant sequences in each simulated dataset was known. The final number of sequences for prevalence analysis and the initial number of sequences with the same DRM had to be same with their difference zero. However, in few amplicons, the number of simulated sequences with a DRM in the final prevalence analysis was observed to be greater than the number of simulated sequence with the same mutation mixed in fixed proportion while creating the data. Those extra numbers of DRMs were created by simulation errors (**Table 4.9**).

Sequencing error was observed to effect at the low expected prevalence level. Few sequences with simulation error had increased the observed prevalence up to twice the expected prevalence (**Table 4.9**).

### 4.3.2.2 Seq2Res web Application Programming Interface (API) and web outputs

Seq2Res web API (available at <http://hiv.sanbi.ac.za/tools/#/seq2res>) has been created for non-bioinformaticians to easy execution of the pipeline for HIV drug resistance test. At Seq2Res homepage (**Figure 4.8**), users have a choice for either submitting a new job or viewing previously submitted jobs.

A click on “Submit job” takes users to Seq2Res job submission page (**Figure 4.9**). Users can provide a job name and upload a raw sequence file, primer file containing the forward and reverse primer details and MID file containing sample specific tags sequences. Users can also set required parameters for the analysis in advanced options and then finally submit the job. Users gets an email in their email ID, used for logging in, after the job processing is completed or error occurred while processing.

A click on “My Jobs” button at Seq2Res homepage takes users to a page containing the list of all the jobs that the user has submitted (**Figure 4.10**). Job details like the name of job, the date of job submission and the status of the job processing - “complete” or “pending” or “error” while processing.

A click on a job from the list of jobs displays two plots that show the overall sequence analysis results of all samples in the input file for that job. The first plot in the result page shows the number of sequences discarded in each step of Seq2Res processing and the number of sequences that are passed in downstream analysis for making final result (**Figure 4.11**). The second plot shows the number of sequences in forward and reverse strands per amplicon per sample that went up to the final stage for producing the final result (**Figure 4.12**).

A list of individual patient results in the form of links are listed down the results page, below the above mentioned two plots. A click on a green “show” button at the left of each patient and MID name takes users to a drug resistance result page of the selected patient.

A table at the top of the sample specific result page shows the “consensus like” drug resistant report of the selected patient using the DRMs with prevalence greater or equal to defined cutoff prevalence (**Figure 4.13**). The susceptibility of drugs for the patient is shown by color code – Red: Resisant, Orange: Intermediate resistant, Green: Sensitive (**Figure 4.13**).

Below the table in a patient specific result page, there are four bar plots – one plot for each drug class – NRTI, NNRTI, PI and Integrase Inhibitors (INs). Each bar plot shows the prevalence of drug class specific DRMs, the DRMs and the coverage of a DRM codon position (**Figure 4.14**).

Back at the bottom of the result page obtained after clicking a job name, there is a link “Further Page”, which takes user to the advanced result page that contains drug resistant report generated using high throughput sequences. A click on the link “Ultra deep drug resistance report” downloads high throughput drug resistance reports of all patients in one PDF file. Each patient specific high throughput drug resistance report is a table that displays antiretroviral drug classes – NRTIs, NNRTIs, PIs or INs in first column, all clinical antiretroviral drugs in second column, the number of sequences analyzed in third column, the percentage of sequences that are resistant to corresponding drug in fourth column, the percentage of sequences that are intermediate resistant to the corresponding drug in fifth column and the DRMs in the sixth column (**Figure 4.15**). The susceptibility of drugs for the patient is shown by color code is same as in “consensus like” drug resistant report.

Below the link for high throughput drug resistant report, there is a link to download the “consensus like” DRMs each patient.

### 4.4 Discussion and Conclusion

HIV drug resistance test is essential to characterize the viral population (Baba et al., 2005; Simen et al., 2009a) and to treat HIV infected individuals with correct combination of antiretroviral drugs to suppress the viral replication for longer time period and increase their life expectancy (Harrison et al., 2010; van Sighem et al., 2010). Roche/454 high throughput sequencing technology has shown great potential to genotype even the minor HIV variants that are in most cases drug resistant variants (Lataillade et al., 2010; Simen et al., 2007; Simen et al., 2009b; Varghese et al., 2009). But Roche/454 sequencing technology produces up to a million sequences ([www.454.com](http://www.454.com)) and manual analysis at any processing step is not feasible. Seq2Res computational pipeline is designed to analyze this huge amount of data for HIV drug resistance test and produce sample specific drug resistance report in both HTS method and Consensus like method; and also show the prevalence of DRMs.

The full-length amplicon sequences that begins from user defined nucleotide “start” position to “end” position are analyzed to consider all the DRMs that the amplicon covers. However, large number of sequences gets discarded although they cover all the DRMs of the amplicon. The DRM codons are usually inwards from the 5’ and 3’ ends in the sequences. The sequences are discarded as not full-length because they don’t cover those non-DRM codon positions at the ends that are not important in the analysis. Therefore, Seq2Res finds the first and last DRM codon positions in the amplicon and considers the sequences covering those positions as optimal full-length. This saves large number of sequences that do not cover non-DRMs codon positions at the ends from being discarded.

Seq2Res DRM calls were compared with the standard tool – web Sierra (Liu and Shafer, 2006) using two sample data. The comparison result was highly congruent; out of over 10683 DRMs in PR and 7867 DRMs in RT genes of sequences in “large data set” and 30765 DRMs in PR and 22569 DRMs in RT genes of sequences in “very large data set”, a single discordant mutation call was observed. However, a close observation in the alignment of the sequence with discordant mutation call and reference sequence showed that Seq2Res mapping tool RAMICS did the alignment correctly in codon space. This showed that Seq2Res was better than web Sierra at DRM call.

Simulated datasets were prepared with known number of simulated sequences containing DRMs. The datasets were analyzed using Seq2Res for the prevalence of the DRMs. Reference mapping of the simulated sequences discarded about 1.8% of PR, 20% of RT1 and 5% of RT2 simulated sequences for all samples at all expected prevalence level (56252’s RT amplicon) as non optimal full-length. Discarding of huge amount of sequences had less effect on the observed prevalence to deviate from the expected prevalence. The observation showed that the observed prevalence of the DRMs in the datasets was very close to the expected prevalence with the mean of observed prevalences from all samples was same as expected prevalence and the standard deviation was close to zero.

Counting the final number of DRMs in the final prevalence analysis and the number of sequences with same DRMs before reference mapping assessed simulation errors in the DRM codon positions. The simulation errors influenced the observed prevalence to be higher than the expected prevalence at the low expected prevalence level.

Roche/454 generates errors at homopolymer region in real sequencing (Gilles et al., 2011). As the simulator tool used the profile of a real sequence data, the homopolymer errors in the simulated data were assessed. The observation showed that for 23% of the sequences, the homopolymer region was sequenced correctly. The chance that simulation generated two insertion errors at homopolymer region was the highest (43.3%) (**Table 4.10).**

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