Video Article

Prediction of HIV-1 Coreceptor Usage (Tropism) by Sequence Analysis using a Genotypic Approach

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Abstract

Maraviroc (MVC) is the first licensed antiretroviral drug from the class of coreceptor antagonists. It binds to the host coreceptor CCR5, which is used by the majority of HIV strains in order to infect the human immune cells (Fig. 1). Other HIV isolates use a different coreceptor, the CXCR4. Which receptor is used, is determined in the virus by the Env protein (Fig. 2). Depending on the coreceptor used, the viruses are classified as R5 or X4, respectively. MVC binds to the CCR5 receptor inhibiting the entry of R5 viruses into the target cell. During the course of disease, X4 viruses may emerge and outgrow the R5 viruses. Determination of coreceptor usage (also called tropism) is therefore mandatory prior to administration of MVC, as demanded by EMA and FDA.

The studies for MVC efficiency MOTIVATE, MERIT and 1029 have been performed with the Trofile assay from Monogram, San Francisco, U.S.A. This is a high quality assay based on sophisticated recombinant tests. The acceptance for this test for daily routine is rather low outside of the U.S.A., since the European physicians rather tend to work with decentralized expert laboratories, which also provide concomitant resistance testing. These laboratories have undergone several quality assurance evaluations, the last one being presented in 2011[1].

For several years now, we have performed tropism determinations based on sequence analysis from the HIV env-V3 gene region (V3)[2]. This region carries enough information to perform a reliable prediction. The genotypic determination of coreceptor usage presents advantages such as: shorter turnover time (equivalent to resistance testing), lower costs, possibility to adapt the results to the patients' needs and possibility of analysing clinical samples with very low or even undetectable viral load (VL), particularly since the number of samples analysed with VL<1000 copies/µl roughly increased in the last years (Fig. 3).

The main steps for tropism testing (Fig. 4) demonstrated in this video:
1. Collection of a blood sample
2. Isolation of the HIV RNA from the plasma and/or HIV proviral DNA from blood mononuclear cells
3. Amplification of the *env* region
4. Amplification of the V3 region
5. Sequence reaction of the V3 amplicon
6. Purification of the sequencing samples
7. Sequencing the purified samples
8. Sequence editing
9. Sequencing data interpretation and tropism prediction

Video Link

The video component of this article can be found at https://www.jove.com/video/3264/

Protocol

The protocol is summarized in Fig. 4.

1. Collection of blood samples

1. Collect at least 3 ml blood in EDTA tubes at the clinical site.
2. The samples can be stored up to a week at 4°C.
3. Send the EDTA-blood samples as soon as possible to the laboratory by ordinary mail.

2. Isolation of HIV RNA and/or DNA

1. Obtain viral RNA and/or DNA from 1000 µl whole blood, serum or plasma, using the MagNA Pure Compact Nucleic Acid Isolation I kit I and the MagNA Pure Compact System.
2. Elute the gained RNA or DNA in 50 µl TE buffer. Alternatively, other nucleic acids purification procedures may be used.

3. Amplification of the env region

Amplify the env region (Figs. 2 and 4) from either plasma RNA or proviral DNA. A 1245 bp-long product, comprising the majority of the Env protein (nt 6556-7811 according to HXB2) is generated.

1. RNA samples: RT-PCR reaction
    1. Viral RNA presents a very complex secondary structure (Fig. 4) that may hinder the RT reaction. To avoid this problem, incubate 10 µl RNA at 65°C for 10 minutes (already in the PCR tube and in the PCR machine) and then reduce the temperature to 50°C.
    2. Add 40 µl of the PCR Master Mix including the primers Env-F and Env-R.
    Master Mix for RT-PCR In-House-System:

	volume/reaction (µl)	final concentration
RNase-free H_2O	14.4	
5x Buffer (Qiagen OneStep RT-PCR Kit)	10	1x
5x Q-Solution	10	1x
dNTP Mix (10 mM each)	2	400 µM of each dNTP
Enzym-Mix (Qiagen OneStep RT-PCR Kit)	2	
Primer Env-F (100 µM)	0.3	0.6 µM
Primer Env-R (100 µM)	0.3	0.6 µM
RNase Inhibitor (RNasin)	1	5 units/reaction

    Env-F: 5'- CAAAGCCTAAAGCCATGTGTAAA -3' (nt 6556→6586 according to HXB2)
    Env-R: 5'- AGTGCTTCCTGCTGCTCCTAAGAACCC -3' (nt 7785←7811)
    3. Run the RT reaction at 50°C for 30 minutes.
    4. Run the first PCR as follows:

95°C, 15min	1 x
95°C, 30 sec 50°C, 30 sec 72°C, 2 min	1 x
95°C, 30 sec 56°C, 30 sec 72°C, 2 min	38 x
72°C, 10 min 4°C ∞	

2. DNA samples: PCR reaction
    For proviral DNA samples, no initial RT reaction is needed, and the PCR reaction can directly start.
    1. Add 40 µl of PCR Master Mix to 10 µl of provial DNA.
    Master Mix for PCR In-House-System:

	volume/reaction (µl)	final concentration
RNase-free H_2O	15.4	
5x Buffer (HotStarTaq DNA Polymerase)	10	1x
5x Q-Solution	10	1x
dNTP Mix (10 mM each)	2	400 µM of each dNTP

Enzym-Mix (HotStarTaq DNA Polymerase)	2	
Primer Env-F (100 µM)	0.3	0.6 µM
Primer Env-R (100 µM)	0.3	0.6 µM

Env-F: 5'- CAAAGCCTAAAGCCATGTGTAAA -3' (nt 6556→6586)
Env-R: 5'- AGTGCTTCCTGCTGCTCCTAAGAACCC -3' (nt 7785←7811)

2. Run the PCR conditions as previously described for RNA samples (Step 3.1.4).

4. Amplification of the V3 region: nested PCR

1. For the nested PCR, use 5 µl from the first PCR reaction (without previous analysis in agarose gel) as template and add 95 µl of PCR Master Mix.
Master Mix for Nested-In-House PCR

	volume/reaction (µl)	final concentration
H_2O	61.9	
10x PCR Buffer	10	1x
5x Q-Solution	20	1x
dNTP Mix (10 mM each)	2	200 µM of each dNTP
Primer Env-2F (100 µM)	0.3	0.6 µM
Primer Env-2R (100 µM)	0.3	0.6 µM
HotStarTaq DNA Polymerase	0.5	2,5 units/reaction

Env-2F: 5'- GTCCAAAGGTATCCTTTGAGCCAATTC -3' (nt 6838→6864)
Env-2R: 5'- CACCACTCTTCTCTTTGCCTTGGTGGGTGC -3' (nt 7712←7742)

2. Run the PCR as follows:

93°C, 15min	1 x
95°C, 30 sec 50°C, 30 sec 72°C, 90 sec	1 x
95°C, 30 sec 56°C, 30 sec 72°C, 90 sec	43 x
72°C, 10 min 4°C ∞	

3. Analyze the PCR product on a 1% agarose gel (Fig. 4). The expected product is 902 bp-long.
4. Purification of the sequencing samples.
    1. Purify the PCR product with the Qiagen PCR-Purification kit, using the protocol, solutions and micro-columns provided by the supplier. Elute in 30-100 µl PE buffer, depending on band intensity.
    2. Alternatively, the samples can be purified using Exonuclease I and Fast AP (Thermo alkaline phosphatase). For this purpose, add 6 µl Exo-AP Mix to 15 µl PCR product and incubate 15 minutes at 37°C.

580 µl H_2O
66 µl fast AP
13.4 µl Exo I

5. Sequence reaction of the V3 amplicon

1. The sequence reaction consists of a PCR reaction using only one of the following primers:
Env-2: 5'- GTACAATGYACACATGGAATTAGGC -3 (nt 6959→6980)
Env-5: 5'- AAAATTCCCCTCCACAATTA - 3' (nt 7352←7371)
Env-6: 5'- GGCCAGTAGTATCAACTCAAC - 3' (nt 6979→7000)
Env-7: 5'- TGTCCACTGATGGGAGGGGC - 3' (nt 7530←7549)
Env-10: 5' - GCAGAATAAAACAAATTATAAACATGTGGC - 3' (nt 7478←7507)
Env-11: 5' - TACATTGCTTTTCCTACTTTCTGCCAC - 3' (nt 7638←7664)
First choice primers are: Env-2, Env-6 or Env-7, Env-11
2. Use 1 µl from the purified V3 amplicon as template and add 9 µl of PCR master-mix.
Sequencing Master Mix:

4 µl sequence mix (HIV-Genotyping Kit)	
4 µl H_2O	
1 µl Primer (100 pmol/µl)	

3. Run the sequence reaction as follows:

96°C, 10 sec	
50°C, 10 sec	36 x
60°C, 45 sec	
4°C ∞	

6. Purification of the sequencing samples

Purify sequences using Sephadex G-50 superfine.

1. Fill the appropriate number of wells in the "column loader" with Sephadex G-50 superfine. Transfer the Sephadex to the filter plate MAHVN4510 by turning over.
2. Add 300 µl Milli-Q H_2O to each well in the filter plate, and incubate it for at least 3h at 2-8°C.
3. Centrifuge the plates for 5 min at 910Xg and 4-15°C. Discard the flow-through.
4. Add 10 µl H_2O to the sequence product and then pipet the sequence on the swelled Sephadex plate.
5. To gain the purified product, centrifuge the plate for 5 min. at 910Xg and 4-15°C and collect the flow-through.

7. Sequencing the purified samples on the sequencer ABI Prism 3130 XL

1. Before each run, change the buffer and check the volume of the polymer (POP7). If needed, replace the old polymer flask by a new one.
2. Use the saved run conditions, including an injection time of 18 seconds.
3. In this machine, the collection of the signal data of one run with 16 sequence samples takes about 60 min (36 cm capillary) or 150 min (50 cm capillary).

8. Sequence editing

1. Use the program Lasergene (DNA-Star) to align and edit the sequences (Fig. 5). Other sequence editing programs may be used. Use a "V3-Consensus B" and the *env* consensus sequence as references.
2. Lasergene creates a consensus sequence of the analysed sample using all the raw data available and store it as FASTA file. The FASTA file is a text file that includes a header with the name of the sample and the nucleotide sequence.

9. Sequencing data interpretation and tropism prediction

1. Sequencing data interpretation is performed by the web-based interpretation system geno2pheno$_{[coreceptor]}$ (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php). For the tropism prediction, different FPR settings can be selected. The default setting is according to the German-Austrian therapy guidelines. For FPR ≥ 20%, the virus is classified as R5 when FPR ≥ 20% and X4 for FPR < 12.5%.
2. Upload the FASTA file into the server. This server translates the nucleotide sequence into amino acids, aligns it with the V3-Consensus B sequence and produces a subtype classification. In addition, it generates a prediction of the coreceptor usage (Fig. 4) expressed as false positive rate (FPR).

10. Representative Results

The geno2pheno$_{[coreceptor]}$ output shows a graded interpretation of the tropism. Depending on the likelihood of the coreceptor usage, the interpretation text and background color varies from green (<20% FPR), suggesting a safe administration for MVC, to yellow, suggesting a possible, low risk and finally to red. Red is the color suggesting not to prescribe MVC. In addition, the server generates a pdf report that can be printed, filled in with patient's and sample data, and sent to the physician. Examples of geno2pheno$_{[coreceptor]}$ output are depicted in Fig. 6.

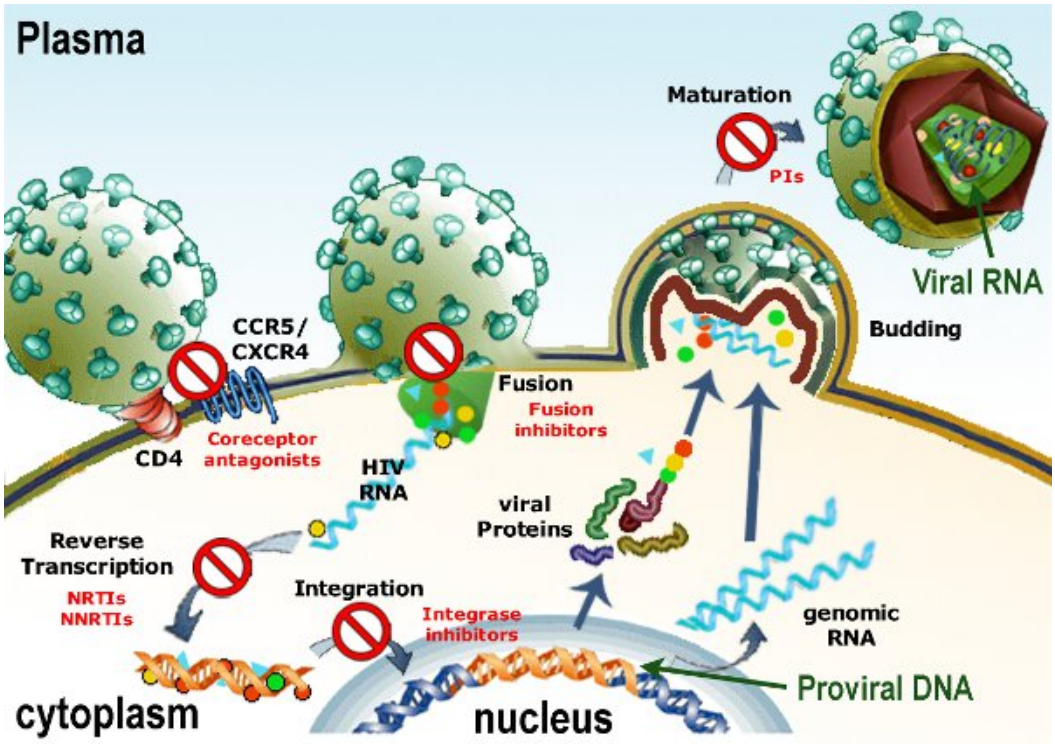

Figure. 1. Schematic replication of HIV. The virion must bind to the cellular CD4 as receptor and to either the CCR5 or CXCR4 as coreceptor. The coreceptor CCR5 can be blocked with CCR5 antagonists like Maraviroc (MVC, Celsentri, Selzentry). After fusion of the viral and cellular membranes, the viral nucleocapsid is released in the cytoplasm. The nucleocapsid disassembles and the viral RNA complex is liberated into the cytoplasm. The viral reverse transcriptase (RT) transcribes the genomic RNA into proviral DNA, that is then transported to the nucleus and integrated into the host genome by the HIV integrase. Cellular RNA polymerases transcribe viral genomic and messenger RNAs from the proviral genome. The viral proteins are produced in the cytoplasm and transported to the cell surface. The virus particles bud as immature, non-infectious virions from the cells. The HIV protease cleaves the proteins producing to infectious particles. Inhibition of one of the steps leads to an interruption of the replication cycle.

The antiretroviral drugs and the replication step that they inhibit are marked in red.

The viral RNA and proviral DNA (material used for tropism or resistance analysis) are marked in green.

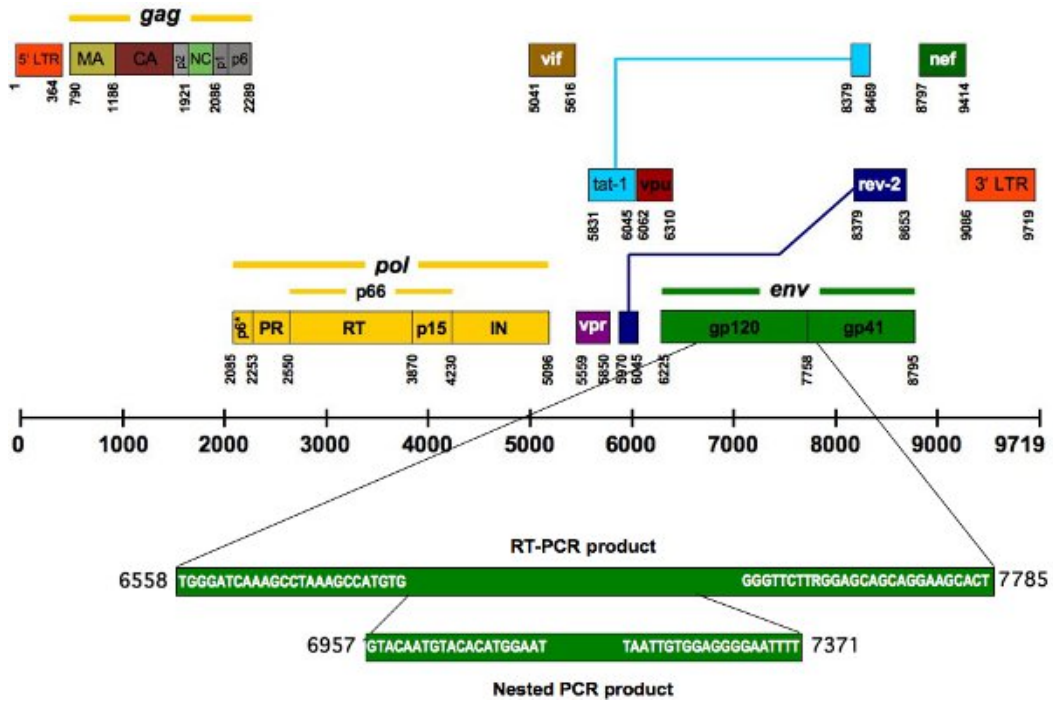

Figure 2. HIV proviral genome. The HIV particle is built with different structural proteins and houses various enzymes and proteins both from viral and cellular origin. The figure shows the location of the genes within the viral genome. The surface proteins gp120 and gp41 constitute spikes on the surface of the virion and contact the human cell to perform the membrane fusion. In the lower part of the figure, the PCR amplification products and the primers used in our protocol are shown.

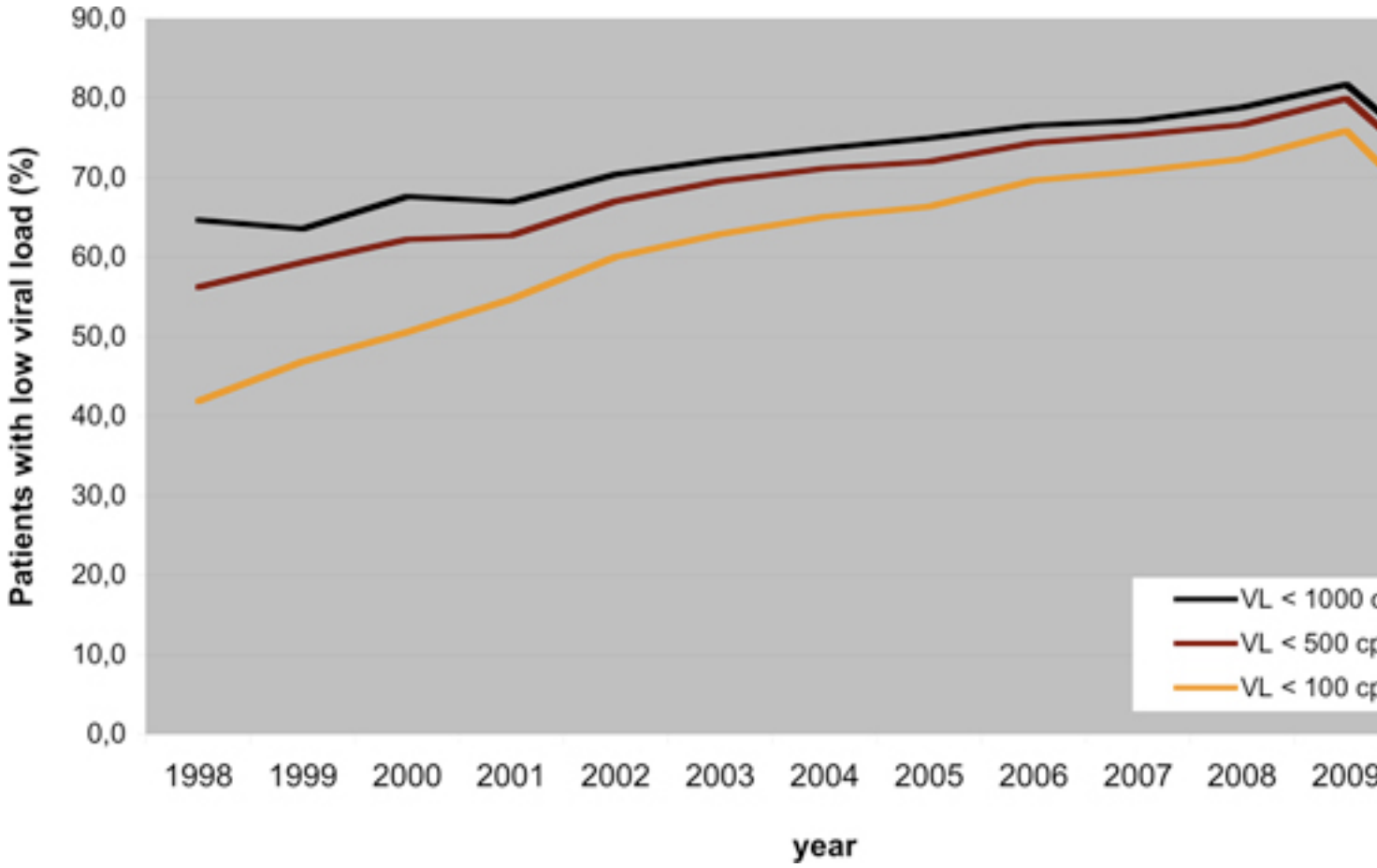

Figure 3. Proportion of patients with low viral load (VL) measurements analyzed for drug-resistance or tropism determination at the Institute of Virology, University of Cologne (Germany).

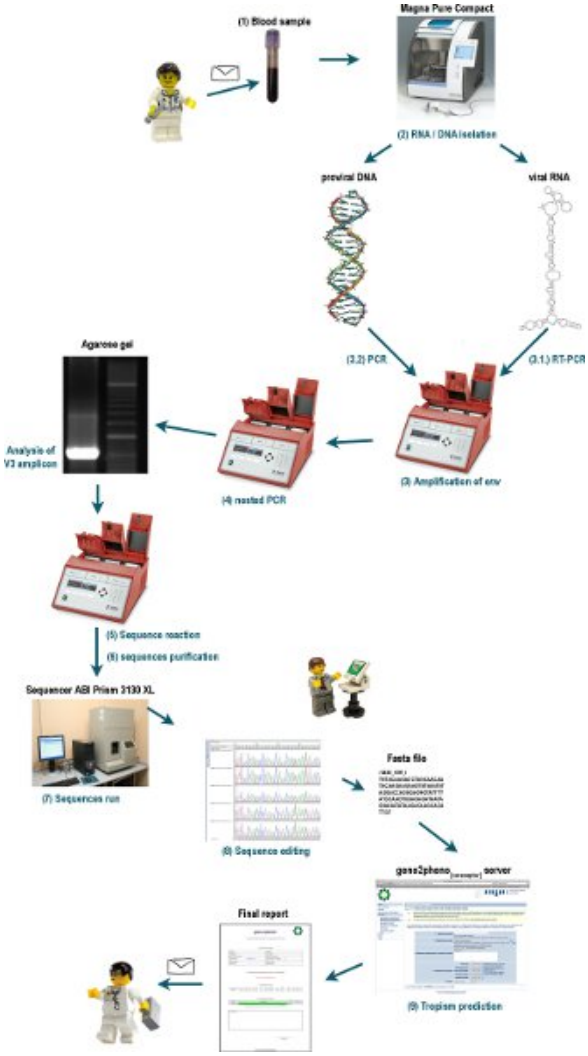

Figure 4. Overall scheme of the experiment.. Please click here to see a larger version of this figure.

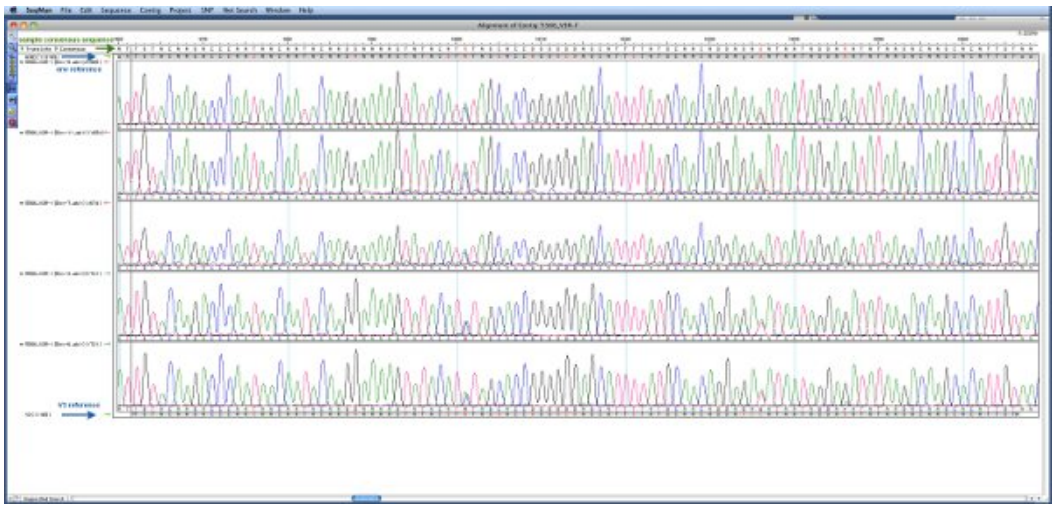

Figure 5. Sequence editing with Lasergene. The V3 amplicon is sequenced with at least one forward and one reverse primer. Lasergene alignes the ".abi" files obtained from the sequencer with the reference sequences "V3-Consensus B" and env. Lasergene creates a consensus

sequence using all the row data available. The reference sequences can be marked (and are then displayed in grey) so that they do not contribute to the sample consensus sequence.

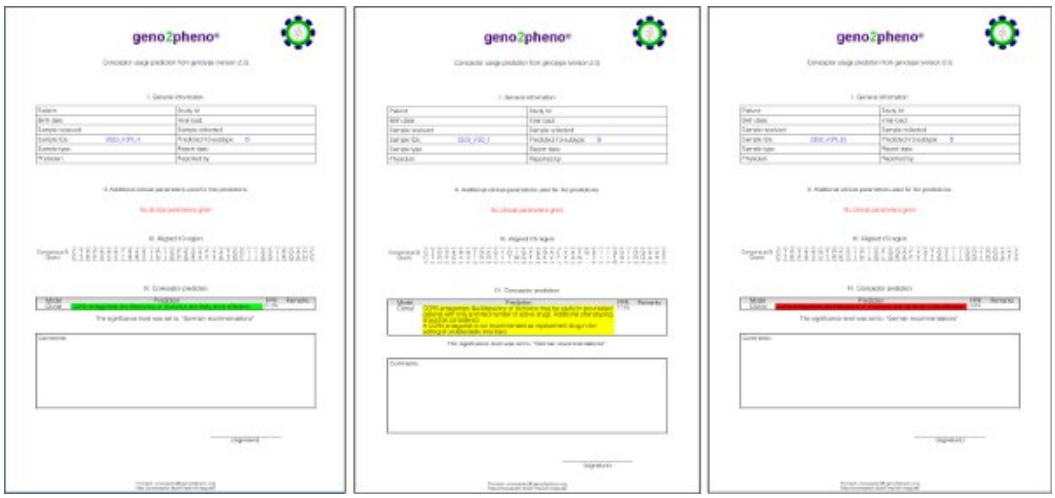

Figure 6. Tropism prediction reports generated by the geno2pheno[coreceptor] tool. The reports are generated as pdf files that can be saved and completed in the computer. Additional data such us patient's name, date of blood extraction, etc., can be manually included using a pdf writer program. Specific comments can be also added. These data are exclusively on the user's computer and not on the geno2pheno[coreceptor] server. Please click here to see a larger version of this figure.

Discussion

The V3 sequence permits a reliable viral tropism prediction, as shown in clinical studies[3-9]. In fact, genotypic determination is contemplated in the current European and German-Austrian guidelines[10].

Compared to phenotypic testing, not only is the turnover time shorter (equivalent to resistance testing), but also are the costs. In addition, a major advantage of genotypic testing is that the results are graded as FPR and therefore can be adapted to the patients' needs. Trofile results, on the other hand, are simply R5 or X4 reports, and access to the raw data is not given.

Currently, The European guidelines suggest a FPR cut-off of 20%[10], while the Austrian-German guidelines allow adapting the FPR cut-off specifically to each patient's needs[11]. In this line, for patients with a broad range of antiretroviral drugs options, higher FPR cut-offs (> 20%) are recommended. Conversely, for heavily pre-treated patients with limited therapeutic options, lower FPR cut-offs (> 5%) may be used. This kind of therapy guidance is currently ongoing by the resistance testing to NRTIs, NNRTIs, PIs, and INIs, where partially-active drugs may be included in the treatment for patients with reduced therapeutic options. In addition, with growing numbers of genotypically-guided therapy changes, the clinically-relevant FPR cut-offs are constantly being adjusted by a panel of bioinformaticians, virologists and clinicians.

Another important advantage of the genotypic tropism testing is the possibility of analyzing clinical samples with very low or even undetectable viral load (VL). In this cases, when plasma RNA is not amplifiable, the proviral DNA can be sequenced and used for reliable predictions[9,12]. Of note, the number of patients with low or undetectable viral load has sharply increased in the latest years. To date, the Trofile assay only allows the analysis of samples from patients with VL<1000 copies/ml. However, preliminary studies have shown that proviral DNA may be also adecuate to be tested by Trofile[13].

Disclosures

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