Development and Performance of Conventional HIV-1 Phenotyping (Antivirogram®) and Genotype-Based Calculated Phenotyping Assay (virco®TYPE HIV-1) on Protease and Reverse Transcriptase Genes to Evaluate Drug Resistance

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Key Words

HIV-1 resistance testing · Genotyping · Phenotyping · Protease inhibitors · Reverse transcriptase inhibitors · Resistance mutations · Linear regression modelling

Abstract

Objectives: A wide array of monitoring tests is commercially available to gauge HIV-1 disease progression and the overall health status of an HIV-1-infected patient. Viral load tests provide a picture of viral activity, while CD4 cell counts shed light on the immune status and can help physicians to prevent the development of opportunistic infections in patients. On the other hand, genotypic and phenotypic resistance testing and therapeutic drug monitoring help to optimize HIV-1 antiretroviral therapy. Resistance testing is currently recommended within the standard of care guidelines to aid the choice of new drug regimens following treatment failure(s). **Methods:** Genotypic testing described here is based on the amplification and sequencing of an HIV-1 protease (PR) and reverse transcriptase (RT) region from a patient sample to identify resistance mutations associated with PR and RT inhibitor resistance. A genotypic test takes a week to perform and the results are reported as a list of detected mutations. The

virco®TYPE HIV-1 report uses genotypic data to predict phenotypic susceptibility by linear regression modeling that uses a large correlative database of genotype-phenotype pairs. Phenotypic testing measures the ability of the virus to replicate in the presence of a drug and provides a direct measurement of drug susceptibility in vitro. Since phenotypic analysis is laborious and time consuming (28 days), genotypic resistance testing is currently the standard reference method used for HIV-1 resistance testing. However, a phenotypic test is important when a patient harbors virus with complex genetic patterns, or when the mutational resistance profile for a particular drug is not well-characterized. **Results and Conclusions:** Some of the currently used resistance tests are partially automated enabling laboratories to increase overall efficiency. However, maximum automation and standardization of the process, instruments and software that we have described here can overcome many of the problems encountered with current tests and aims at having a compliant, high-throughput, diagnostic laboratory, which can guarantee sample integrity from sample reception to result reporting. We also describe in detail the development and performance of virco®TYPE HIV-1 (genotype) and Antivirogram® (phenotype) assay on PR and RT genes to evaluate antiretroviral resistance.

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Introduction

HIV-1 drug resistance testing has been shown to improve antiretroviral treatment outcomes and is broadly recommended as standard-of-care in current treatment guidelines, particularly for patients with prior treatment experience [Panel on Antiretroviral Guidelines for Adults and Adolescents. Department of Health and Human Services, 2011;1–167. Available at <http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>]. Resistance testing may be performed by direct measurement of virus susceptibility to drugs (phenotyping) or through analysis of the sequence of genes encoding antiretroviral targets (genotyping). Phenotyping measures resistance directly but is relatively slow and expensive compared to genotypic analysis, while genotypic data may be complex and its interpretation requires a priori knowledge of the impact of mutations (including their mutual interactions) on drug susceptibility. A phenotypic test can be most helpful when a patient harbors virus with complex genetic patterns, or when the mutational resistance profile for a particular drug is not well-characterized. A third method is a hybrid approach or system which uses genotypic data to predict phenotypic susceptibility. This method requires a large database of paired geno- and phenotypes but predicts phenotypic susceptibility as rapidly as genotypic testing and with comparable accuracy [1] and specificity compared to the actual laboratory-performed phenotype.

Currently, physicians thus have the choice to use genotypic tests (with or without the hybrid approach) which provide a qualitative resistance interpretation based on algorithms derived by experts, or to use phenotypic tests which provide a quantitative resistance interpretation (comparison of the level of inhibition of the patient's virus with that of a virus known to be fully susceptible).

The virco[®]TYPE HIV-1 resistance interpretation system for protease (PR) and reverse transcriptase (RT) inhibitors utilizes the third methodology mentioned above and is based on *VirtualPhenotype-LM*, a system that combines several of the advantages of genotypic and phenotypic drug resistance testing. It accurately predicts phenotypic susceptibility from a genetic sequence based on linear regression modeling using a large (>80,000 paired samples) database of genotype-phenotype pairs [2, 3]. On the other hand, the phenotypic recombinant virus assay for susceptibility testing of RT inhibitors was first described [4], with subsequent modifications towards susceptibility testing of PR and RT inhibitors reported by Hertogs et al. [2].

Since the initial description of the genotyping and phenotyping assay performed at Virco, a number of laboratory modifications and improvements have been made to the assays. The purpose of the present paper is to provide a summary update of the current assays, including a description of the methodology and the characteristics used for processing viral isolates in a controlled, diagnostic, high-throughput setting.

Materials and Methods

Cell Lines, Virus Strains, and Compounds

MT4 Cells

The human T lymphoblastoid cell line MT4 was kindly provided by Dr. Masanori Baba (Center for Chronic Viral Diseases, Kagoshima University, Kagoshima, Japan). All cells are cultured in RPMI 1640 medium Biowhittaker (Walkersville, Md., USA) supplemented with 10% FCS (Lonza, Verviers, Belgium) and 0.02% gentamicin (Invitrogen, Carlsbad, Calif., USA) in Corning ProCulture spinner flasks (Corning, Corning, N.Y., USA; referred to as spinner bottles) in a humidified incubator with a 4.5% CO₂ atmosphere at 37°.

Construction of MT4-LTR-EGFP Cell Line

The pLTR-EGFP plasmid was constructed by replacing the CMV promoter from the pEGFP-C1 plasmid (BD Biosciences Clontech, Mountain View, Calif., USA) with a PCR-amplified 5' long terminal repeat (LTR) promoter from pHXB2D provirus (ARP206; NIBSC, South Mimms, UK), flanked by *Asel*/*AgeI* restriction enzyme sites. The MT4-LTR-EGFP cell line was then constructed by Pfx 2 (Invitrogen) lipid transfection of MT4 cells with pLTR-EGFP. Transfected cells were selected with geneticin (G418, Invitrogen, Carlsbad, Calif., USA), the mammalian selection drug for the neomycin resistance gene. To this end, cells were cultured for 3 weeks in RPMI 1640 without phenol red, but supplemented with 1% Ultra Glutamine (Biowhittaker), 10% FCS (HyClone, South Logan, Utah, USA), 20 µg/ml gentamicin and 800 µg/ml G418. Only 10% of the surviving cells did express EGFP. These cells were isolated by fluorescence activated cell sorting (FACS) using a FACSCalibur (Becton Dickinson, Erembodegem, Belgium). The cell line with the highest EGFP expression was cloned. This cell line displays an HIV-1-Tat-inducible EGFP expression and was stored at –80° or maintained in RPMI 1640 supplemented with 10% FCS, 0.02% gentamicin and 0.8% G418.

Viral Strains

The wild-type HIV-1 strain IIIB was kindly provided by Dr. G. Van der Groen (Institute for Tropical Medicine, Antwerp, Belgium). HIV-1 site-directed mutants were ordered at Medigenomics (Munich, Germany). The wild-type HIV-1 strain HXB2 is used as the reference virus for nucleotide sequence alignment of the PR-RT genes for virco[®]TYPE HIV-1 assay.

Antiretroviral Compounds

The reference compounds zidovudine (AZT), lamivudine (3TC), didanosine (ddI), stavudine (d4T), abacavir (ABC), emtricitabine (FTC), tenofovir (TDF), nevirapine (NVP), efavirenz

(EFV), etravirine (ETR), indinavir (IDV), nelfinavir (NFV), saquinavir (SQV), amprenavir (APV), lopinavir (LPV), atazanavir (ATV), tipranavir (TPV) and darunavir (DRV) were received from the corresponding pharmaceutical companies or purchased from commercial sources.

Plasma Samples

Blood samples of the patients were collected in ethylenediaminetetraacetic acid tubes, citrate tubes, or plasma preparation tubes (PPT™) and the samples had to be centrifuged within 8 h following collection. Plasma samples were immediately frozen at -80° and thawed no more than 3 times after having been received and aliquoted within the laboratory. Plasma samples were shipped on dry ice. Written informed consent for study participation and/or for research analysis was obtained from all patients.

Viral RNA Extraction

RNA was extracted from 256 μ l of plasma on the automated RNA extraction platform, BioRobot MDx Workstation (Qiagen, Cat. No. 900600, Hilden, Germany), with a final RNA elution in 65 μ l of H_2O . If no amplification product was obtained using this MDx RNA, the RNA extraction was repeated in 600 μ l of plasma using the semiautomated EasyMAG (EasyMAG, bioMérieux, Boxtel, The Netherlands), with elution in 25 μ l of H_2O .

Amplification of 1,997 bp PR (Codons 1–99) and RT (Codons 1–400) Coding Sequences

The *Gag*(p7/p1-p1/p6)-PR-RT coding sequence, containing the downstream part of *Gag* and about two thirds of the adjacent 5'-end of the polymerase gene (POL) region, was reverse transcribed and amplified in a one-step RT-PCR (35 μ l final volume, Superscript III HF, Invitrogen) using PRTO5 [1] and OUT3 [1], followed by a nested PCR (Expand High Fidelity Polymerase, Roche), using 3'-In, 5'-CATCTACATAGAAAGTTTCTGCTCC-3' and 5'-In, 5'-CTAGGAAAAAGGGCTGTTGGAAATG-3'. Negative and positive controls were included in each run.

Purification of Amplified Coding Sequences

All amplicons were purified (Qiaquick 96 PCR Purification Kit, Qiagen, Hilden, Germany), analyzed, and quantified by agarose gel electrophoresis using Fuji LAS (Fuji, Düsseldorf, Germany) and Aida Image Analyzer v.4.14.

Construction of Proviral Clone pGEMT3ΔPR-RT

The pGEMT3ΔPRT [1] was modified on both sides of the *Bst*EII restriction site (fig. 2). The pGEMT3ΔPRT vector was linearized using *Apa*I, treated with Klenow enzyme and subsequently digested with *Bal*I, removing a 707-bp fragment. This fragment was replaced by a *Bal*I-digested amplicon generated from the initial HXB2-D clone [5] using the oligonucleotides AmplF (5'-CCTAGGAAAAAGGGCTGTTGGAAATGTGGAAAGGAA-GGTACCTGGATTCTGAGTGGGAG-3') and AmplR (5'-CCGTAGCACCGGTGAAATTGCTGCC-3'). The initial HXB2-D clone amplicon was 872 bp long and was digested with *Bal*I, removing the 5' 52-bp tail. This digested amplicon was ligated in the pGEMT3ΔPRT (*Apa*I, *Bal*I) vector resulting in an HIV-1 clone lacking the last 242 bp from *Gag* (G), PR and all but the last 93 bp of RT. The *Bst*EII restriction site used to linearize the vector prior to cotransfection with the GPRT amplicon (see below) was incorporated in the first primer (underlined).

Cotransfection of PR-RT Coding Sequences with pGEMT3ΔPR-RT

MT4 cells were subcultured at 600,000 cells/ml density on the day of transfection. Cells were resuspended in Solution V (Lonza, Cologne, Germany) at a concentration of 2.5×10^6 cells/100 μ l. Transfections were performed with a mixture of 1 μ l of the *Bst*EII-linearized pGEM3ΔGPRT vector (1 μ g/ μ l) and 9 μ l of the purified nested PCR-amplified fragment of ~ 1.9 kb (containing the GPRT sequence derived from the plasma virus population of the patient) using an Amaxa Nucleofector II Pulser. The suspension of transfected cells was transferred into 10 ml of culture medium (RPMI medium supplemented with 10% FCS and 0.02% gentamicin) and incubation was performed at 37° in a humidified 4.5% CO_2 atmosphere. Cell cultures were monitored for the appearance of a cytopathic effect induced by infectious virus replication and harvested by centrifugation.

Antiviral Drug Susceptibility Testing (Phenotyping, Antivirogram®)

Recombinant virus stocks obtained after transfection were titrated using a 1:2 dilution series (8 dilutions) of virus stock that was added to MT4-LTR-EGFP cells in a 384-well plate following the procedures described below (but omitting addition of antiviral drug).

Freshly thawed MT4-LTR-EGFP cells were cultured in Procul-ture spinner flasks (Corning, referred to as spinner bottles), and they were kept in suspension by the use of a magnetic stirrer at a tip speed of 10 inch/s. The bottles contained a cell suspension with a starting concentration of 100,000 cells/ml; percentage viability and cell density were measured with a Beckman Coulter Vi-cell-automated instrument (Analisis, Gent, Belgium).

Cell lines need to be maintained in G418 (800 μ g/ml) for stable expression of EGFP. G418 was omitted from the medium prior to use in antiviral susceptibility experiments.

Drug susceptibility was measured in 384-well black view plates (Corning). Four-fold dilutions were used for all commercially available antiretroviral compounds as well as for compounds under development (10 μ l/plate). The range of dilutions used for each drug was a function of the IC_{50} value of the respective compound. In the next step, MT4-LTR-EGFP cells (670,000 cells/ml), virus, and medium (containing 10% FCS) were mixed and added to the wells (30 μ l/well) using a Hamilton Liquid Handler (Hamilton, Bonaduz, Switzerland). The control wells (for virus and cell) were filled appropriately. Afterwards, the plates were incubated for 72 h in a conditioned, humidified environment (37° , 4.5% CO_2).

After 3 days, the wells were examined for EGFP expression using a modified Zeiss Axiovert 200M (Zeiss, Zaventem, Belgium) fluorescent microscope. The results were expressed as IC_{50} values defined as the concentration of compound achieving 50% inhibition of the virus-induced EGFP signals as compared to the untreated virus-infected control cells. The ratio between the plasma isolate IC_{50} and the wild-type reference virus (IIB) IC_{50} gives the fold-change value, an indicator of the level of resistance attained by the patient isolate against each specific drug.

Dideoxynucleotide-Based Sequence Analysis (Genotyping)

Dideoxy sequencing reactions were performed on the purified amplicon (ABI Prism Big Dye Terminator Cycle Sequencing Kit, Version 3.1, Applied Biosystems) with a set of eight sequence-spe-

cific primers distributed over the PR-RT sequence for both strands: F1, 5'-GAGAGCTTCAGGTTTGGGG-3'; F2, 5'-AATTGGGCCTGAAAATCC-3'; F3, 5'-CCTCCATTCCTTTGGATGGG-3'; F5, 5'-CACTCTTTGGCAACGACCC-3'; R1, 5'-CTCCC-ACTCAGGAATCC-3'; R3, 5'-CTTCCCAGAAGTCTTGAGTTC-3'; R5, 5'-GGGTCATAATACTCCATG-3'; R6, 5'-GGAA-TATTGCTGGTGATCC-3'. Reactions were purified using a DyeEx Purification Protocol (Qiagen), and analyzed using the ABI 3730xl DNA Analyzer (Applied Biosystems).

Sequence data files were grouped per sample identifier (ID) and aligned against the reference HXB2 reference sequence by means of the Sequencher™ Program V 4.1.4 (Gene Codes Corp.). Using the reference sequence as an aid, the data were trimmed to size and points of ambiguity were checked by visual observation of the associated chromatograms to obtain a consensus sequence. A 25% scoring rule was used to define the mixtures observed and this mixture scoring procedure was compared head to head with the 454 deep sequencing to evaluate the frequency of mixture scoring between the 2 sequencing methodologies [6]. The validated sequence from the reviewed data were exported for resistance analysis using *virco*®TYPE HIV-1.

Sequence Interpretation (virco®TYPE HIV-1)

A bioinformatics tool based on linear regression modeling applied to matched genotype/phenotype data contained in a large database was developed to calculate predicted fold-change values from any HIV-1 sequence that is generated as described by Vermeiren et al. [3]. The fold-change interpretation needs to be put into a clinically relevant context in order to enable a clinician to make treatment decisions: whether a viral strain is susceptible or resistant to an antiretroviral agent, or whether a clinician should expect maximum activity or response from a drug versus minimum activity or response. In the *virco*®TYPE HIV-1 system, fold changes are interpreted in function of resistance or susceptibility to an antiretroviral agent (using laboratory-based biological cutoffs) or in function of expected response to an agent in combination therapy (using clinical cutoffs, where a large data set of viral sequences from patients with known clinical outcomes is available) [7].

Results

The processes of phenotyping and genotyping HIV-1 from patient samples (fig. 1) are based on Hertogs et al. [2] with the adaptations described below.

RNA Extraction and PR-RT Amplicon Generation

Viral RNA extraction evolved from manual or robotic extractions using the QIAamp Viral RNA Extraction Kit towards MDx extraction (Qiagen) of 256 µl plasma as first line in combination with a sensitive EasyMAG-based RNA extraction procedure [8], starting from larger plasma volumes (600 µl). Additional retrospective analyses of plasma samples that were either hemolytic [9], lipemic/icteric [10] or HCV coinfecting [11] indicated that plasma

anomalies did not have an impact on the success rate or quality of amplicon generation and results of geno-/phenotyping.

The procedures to amplify the PR-RT-coding sequences evolved from separate reverse transcription using Expand Reverse Transcriptase (Roche) followed by a nested PCR to a combined one-step RT-PCR and a nested PCR. Outer primers used in the one-step RT-PCR were identical to the primers used previously (PRT05 and OUT3) [2], while the nested PCR amplification primers were replaced by 5'-In and 3'-In, generating a final 1,868-bp PR-RT amplicon.

The success rate of PR-RT amplicon generation and the influence of MDx/EasyMAG extraction and combined RT-PCR procedures thereon were analyzed independently by the Chelsea and Westminster Hospital [12] on 112 samples with a viral load (VL) as low as 50 copies/ml. In addition, performance of the EasyMAG platform was also tested on 145 patient samples with VL ranging from 50 to 10,000 copies/ml and a 100% success rate was obtained for samples with VL ≥400 copies/ml [8]. The chance of having a successful amplification result as compared to the earlier setting increased from 90.3 to 95.8% (95% confidence interval, $p < 0.0001$). A retrospective 12-year analysis of a large sample cohort with externally determined (VL >135,000 processed samples) indicated that the success rate increased when VL was ≥1,000 copies/ml (95% or more) and a 57% success rate was obtained for samples with VL ≤1,000 copies/ml [13]. The majority of samples (>75%) analyzed were clade B and the derived amplicon was successfully genotyped and/or phenotyped.

Phenotyping (Antivirogram)

Firstly, the proviral clone pGEMT3ΔPRT used for phenotyping [2] was replaced by the HIV-1 clone pGEMT3ΔGPRT lacking the last 242 bp from *Gag*, complete PR and all but the last 93 bp of RT. This new approach enabled the study of the *Gag* cleavage sites p7/p1 and p1/p6 but also ensured a greater success in creating a functional recombinant virus after transfection compared to the backbone described by Hertogs et al. [2] (data not shown).

Secondly, BioRad electroporation (transfection) procedure [2] was substituted by Amaxa nucleofection, which introduces the genetic material directly into the nucleus. Virus stocks were harvested at the appearance of cytopathic effect, usually 8–9 days (maximum 11 days) posttransfection in the BioRad procedure. A comparison between Amaxa and BioRad, using 50 randomly selected

Fig. 1. Process flow for genotyping and phenotyping of plasma samples.

Fig. 2. Structure of the clone pGEMT3ΔGPRT lacking the last 242 bp from *Gag*, complete PR and all but the last 93 bp of RT as basis for the phenotyping assay [2].

amplicons, demonstrated that virus stocks generated with Amaxa nucleofection can be harvested from day 5 onwards (3–4 days earlier in comparison to BioRad transfection).

Thirdly, as opposed to the published MT4-MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [2], antiviral susceptibility was determined on MT4-LTR-EGFP cells. The MT4-MTT assay measures MT4 cell viability via mitochondrial transformation of the added yellow MTT to purple formazan [14] and evaluates virus infection and protection of cells by antiviral drugs in an indirect way. In contrast, the MT4-LTR-EGFP assay, HIV-1 Tat present in infected MT4 cells, induces the LTR-controlled production of EGFP which enables a direct detection of infection. As a result, the detection of viral infection is more specific, incubation times are considerably reduced (from 5 days for MT4-MTT to 3 days for MT4-LTR-EGFP) and fewer action steps are required to perform the latter assay.

Finally, antiviral susceptibility testing was up-scaled to a high-throughput environment using Hamilton liquid handlers and 384 flat-bottomed Blackview plates (replaced the transparent 384 plates). As a result, 16 antiretroviral compounds can be tested on two 384-well plates instead of 11 compounds on 96-well plates while using the earlier assay [2]. The implementation of the liquid handlers and Blackview plates resulted in increasing the efficiency, significantly decreased the percentage repeat analysis (from 35–40 to 15–20%) and reduced the assay variability due to automation replacing manual handling.

With regard to phenotyping (Antivirogram), there are no external proficiency testing programs available to date, to support this method of testing. Therefore, an internal phenotyping (inclusive genotyping) proficiency testing is executed with a well-characterized panel of five recombinant virus stocks, which demonstrates resistance for drugs or drug classes and is tested on a quarterly basis. Biological test cutoffs, separating HIV-1 strains with a normal range of susceptibility from viral strains with reduced susceptibility, were determined for the Antivirogram assay (Version 2.5.01) by defining the 97.5th percentile of the fold-change values determined in vitro on genetically wild-type viruses [1].

Genotyping (Sequencing Process)

A Gene Amp 9700 Thermocycler, with the reagents from the ABI Prism Big Dye Terminator Cycle Sequencing Kit (Version 3.1), was used to determine the dideoxynucleotide sequence of the GPRT amplicon. The reac-

tions were purified using DyeEx (Qiagen) Purification Protocol. The dye-labeled DNA fragments were separated according to size using ABI 3730xl DNA Analyzer.

The obtained sequence data files were grouped per sample identifier and aligned alongside the reference HXB2 sequence using the Sequencher™ Program (Version 4.1.4). A 25% mixture scoring rule (similar to 20% mixture identification by 454 deep sequencing) was used for the electropherogram analysis [6]. Changes from the reference sequence were examined for their possible association with antiretroviral drug resistance using drug-defined clinical cutoffs and/or biological cutoffs with linear regression modeling to generate a virco®TYPE HIV-1 (Version 4.3.01) report [1, 3, 15, 16].

The accuracy and interassay reproducibility of the genotyping assay was demonstrated by the successful identification of all mutations within a defined panel of 7 clinical isolates tested on a quarterly basis over a period of 7 years [17]. In addition, participation within an external CLIA-approved proficiency testing program (AccuTest HIV-1 PR-RT genotyping testing, Accrometrix) has resulted in successful 6-monthly rounds for the last 10 years.

To investigate prospectively the ability to generate amplicons from samples of different HIV-1 subtypes, two panels of HIV-1 samples with known clade and VL were purchased from Biomedical Business International Diagnostics and processed. It could be concluded that based on these experiments, the current amplification primers had no problem in analyzing HIV-1 group M clades A, B, C, D, CRF01_AE, F, CRF02_AG, G, and H from the commercial panel. This experimental work also showed that the current protocols were able to successfully generate an amplicon for varying dilutions with clades A, B, C, D, CRF01_AE, F, CRF02_AG, G, and H (n = 9). With regard to HIV-1 group O samples, success in amplification was only attained in one of the clade O serial dilutions (table 1).

Sequence Interpretation (virco®TYPE HIV-1)

Interpretation of sequences generated using the genotypic methods described above is carried out by using the virco®TYPE HIV-1 system (virtual phenotype). The system uses a correlative database of geno- and phenotype paired samples which are continuously updated with both clinical trial and patient cohort data. Therefore, over time, the clinical and biological cutoffs might change as new treatment regimens are introduced in clinical practice. The system demonstrated high correlation with treatment outcomes when the predictions are evaluated in unseen validation data sets from the clinical

Table 1. Amplicon recovery rate per extraction method (MDx or EasyMAG) as a function of clade and viral load

Panel ID mother tube	Sample nature	Panel HIV subtype	Panel viral load copies/ml	Amplicon recovery after MDx extraction	Amplicon recovery after EasyMAG extraction
PRD202-01	plasma	A	2×10^5	+	+
PRD202-02	plasma	B	1×10^5	+	+
PRD202-03	plasma	C	5×10^4	+	+
PRD202-04	plasma	D	1×10^5	+	+
PRD202-05	plasma	CRF01_AE	4×10^4	+	+
PRD202-06	plasma	F	1×10^5	+	+
PRD202-07	plasma	CRF02_AG	1×10^5	+	+
PRD202-08	plasma	G	7×10^4	+	+
PRD202-09	plasma	H	1×10^5	+	+
PRD202-10	plasma	negative control	negative control	–	–
PRD301-01	plasma	O	3×10^2	–	–
PRD301-02	plasma	O	2×10^3	+	+
PRD301-03	plasma	O	8×10^2	–	–
PRD301-04	plasma	O	3×10^2	–	–
PRD301-05	plasma	negative control	negative control	–	–

outcome database [18]. Baseline sensitivity scores for treatment regimens within this clinical outcome data set ($n = 2,108$ treatment change episodes, virco® TYPE HIV-1 version 3.8.00) were correlated with a response rate at weeks 8 and 24 using logistic regression accuracy (area under the ROC curve). Values ranging from 0.63 (when missing data are considered treatment failures) at 24 weeks of treatment to 0.79 (when missing values are censored at 24 weeks) or when analyses were done at 8 weeks of treatment were obtained. In comparison with other interpretation systems, these values represent a high correlation with outcome, e.g. for the ANRS system the ranges of the area under the ROC curve are between 0.63 and 0.75 [18].

Discussion

The use of highly active antiretroviral therapy for the treatment of HIV-1 infection has led to a remarkable increase in survival of HIV-infected individuals [19]. However, due to a combination of extremely high levels of virus production and a high mutation rate, HIV-1 resistance to antiretroviral drugs is increasing, making therapy less effective over time [20]. Therefore, close monitoring of HIV-1 resistance is required and in order to achieve this goal, plasma samples from patients were analyzed by genotypic and/or phenotypic means.

The genotyping method employed here (virco®TYPE HIV-1) is based on population-based DNA sequencing of

the viral genome coding for PR (amino acids 1–99) and RT (amino acids 1–400). This 1,868-bp-long region encompasses the genetic loci at which mutations associated with resistance to currently commercially available PR and RT inhibitors.

The basic principle of our phenotypic test (Antivirogram) is the construction of chimeric HIV-1 strains composed of the PR-RT sequences isolated and amplified from the patient's viral RNA, recombined inside CD4+ T cells with an HIV-1 DNA construct from which the PR/RT sequences have been deleted (pGEMT3ΔGPRT). The procedure does not involve any cloning steps and attempts to create a panel of recombinant HIV-1 strains whose diversity reflects the population of virus sequences circulating in the patient. The advantage of using laboratory virus strains for recombination is that these syncytium-inducing strains can be used to infect CD4+ T cell lines (vs. variable sources of donor lymphocytes) and that these viruses grow faster and to higher infectious titers. The turn-around time of the phenotypic assay described here is approximately 4 weeks.

Several adaptations have been made since the previous report on our process [2]. These process modifications were driven by technological advances and by the results of our research.

Changes in RNA extraction and PR-RT amplicon generation led to a robust high-throughput process, with successful amplicon generation being observed in 95.8% of the cases as compared to only 90.3% in the earlier setting [2]. These changes were driven by a profound automation

of the RNA extraction from plasma samples (MDx/EasyMAG) and the introduction of a combined RT-PCR process with new nested PCR amplification primers (5'-In and 3'-In).

As to the phenotyping protocol, almost every step has been upgraded and up-scaled. The replacement of proviral clone pGEMT3ΔPRT [2] by pGEMT3ΔGPRT enabled the study of *Gag* cleavage sites p7/p1 and p1/p6 and led to a more successful creation of functional recombinant virus after transfection. Amaxa nucleofection instead of the former BioRad method [2] resulted in a 3- to 4-day faster nucleofection step. The colorimetric MT4-MTT assay to determine viral drug susceptibility was replaced by the fluorimetric MT4-LTR-EGFP assay, enabling direct detection of infection via viral induction of EGFP production. The homogeneous MT4-LTR-EGFP assay facilitates more specific detection of viral infection, miniaturization and reduction of incubation times, and can be performed in fewer steps. During the optimization of the protocols, liquid handlers were implemented to automate the antiviral experiment process and transparent 384-well plates were replaced by Blackview 384-well plates with a transparent flat bottom. These plates are specially designed for optical clarity in read-through applications, reducing background signals. It was demonstrated that EGFP signals from infected cells were homogeneously spread throughout the well suggesting improved virus infectivity in the confluent layer of cells in Blackview plates as compared to transparent plates. As a result, the need for repeat analysis and the variability of the susceptibility testing assay were significantly decreased.

The goal of upgrading our genotyping and phenotyping assay instruments and softwares is to have a compliant, high-throughput laboratory series of platforms and processes that can guarantee sample integrity from sample reception to result reporting. The performance characteristics and validations required for HIV-1 genotyping and phenotyping have been determined within the laboratory and have been published. Using barcode readers along with compliant, validated softwares for handling readouts and subsequent verification algorithm, the risk of sample switches during the laboratory process has been significantly reduced and sample integrity is ensured.

Genotype-based resistance determinations have shorter turn-around times than phenotypic tests. Moreover, the concordance between susceptibility predictions based on genotypic data and actual virologic response in treated patients is good [3]. However, given the abun-

dance of HIV-1 mutations and the complex relationships between them, making the right predictions is not always straightforward. Interpretation with respect to drug resistance of changes at the relevant loci may be made by means of a set of predefined rules established by experts or by a data-driven system [21]. The Virtual Phenotype engine developed at Virco has evolved from predicting resistance based on the measured phenotype of viruses that have the same mutational profile as the virus being sequenced to the more sophisticated current virco®TYPE HIV-1 system where each individual mutation deviating from the reference sequence is given a specific resistance weight factor calculated using bioinformatics and biostatistics tools applied in a very large database containing paired sequence and phenotype information. In addition, the use of this generated fold-change value in the context of a clinical outcome data set has made possible the development of clinically relevant cutoffs that have over time shown very good performance in predicting virologic outcome when compared with rules-based systems such as the ones employed by the ANRS, Stanford University, REGA and other commonly utilized genotypic interpretation tools.

Both genotypic and phenotypic resistance testing have a role in clinical practice. Whereas genotype is a more straightforward and practical test to perform, phenotype might be required to explain and predict some treatment failures. In addition, phenotyping is indispensable in identifying previously unknown HIV-1 resistance-associated mutations such as K66N in RT that reduces susceptibility to both 3TC and FTC [22], which is needed to improve current interpretation systems. Virtual phenotyping represents an option to obtain phenotypic information generated from only genotypic sequencing. It offers the advantage that any HIV-1 PR-RT sequence that might be generated locally at a reference laboratory can be submitted through the informatics tool to generate a phenotypic interpretation report within minutes.

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