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Analysis of HIV-1 Reverse Transcriptase Activity in Plasma: A New Tool for the Detection of Viral Variants, Virus Load Measurement, and Phenotypic Drug Resistance Testing

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Abstract

Detection of reverse transcriptase (RT) activity in plasma is now possible through the use of ultrasensitive RT assays. These assays are up to 1 million-fold more sensitive than conventional RT assays because of the use of PCR amplification for detecting the RT reaction product. One of these assays, named Amp-RT, has been demonstrated to detect RT from retroviruses representing different genera. The ability of Amp-RT to detect RT activity in small volumes of unprocessed plasma samples provides a rapid tool for the generic detection of known or novel retroviruses in biological fluids. Quantitative detection of RT activity by Amp-RT has also been developed, providing a functional marker for plasma virus load analysis in HIV-1 infections. Levels and kinetics of RT- and RNA-based virus loads correlate with each other. However, significant variabilities in RT/RNA ratios have been observed among patients. The Amp-RT assay has also been adopted for screening plasma for phenotypic resistance to several HIV-1 RT inhibitors such as 3TC and nevirapine. The RT-based drug susceptibility results correlate with those determined by replication-based assays and with the detection of genotypic markers of resistance, thus providing a rapid new approach for clinical monitoring of phenotypic drug resistance. RT analysis by the Amp-RT assay provides a simple, rapid, and sequence independent tool for the detection of viral variants, virus load measurements, and phenotypic drug resistance testing.

Key words

Reverse transcriptase. Amp-RT. Virus load. Drug resistance.

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Introduction

Retroviruses are widely prevalent among vertebrates and are the causative agent for a variety of diseases in humans including immunodeficiencies, leukemias, and lymphomas[1]. The retrovirus family is characterized by the presence of particle-associat-

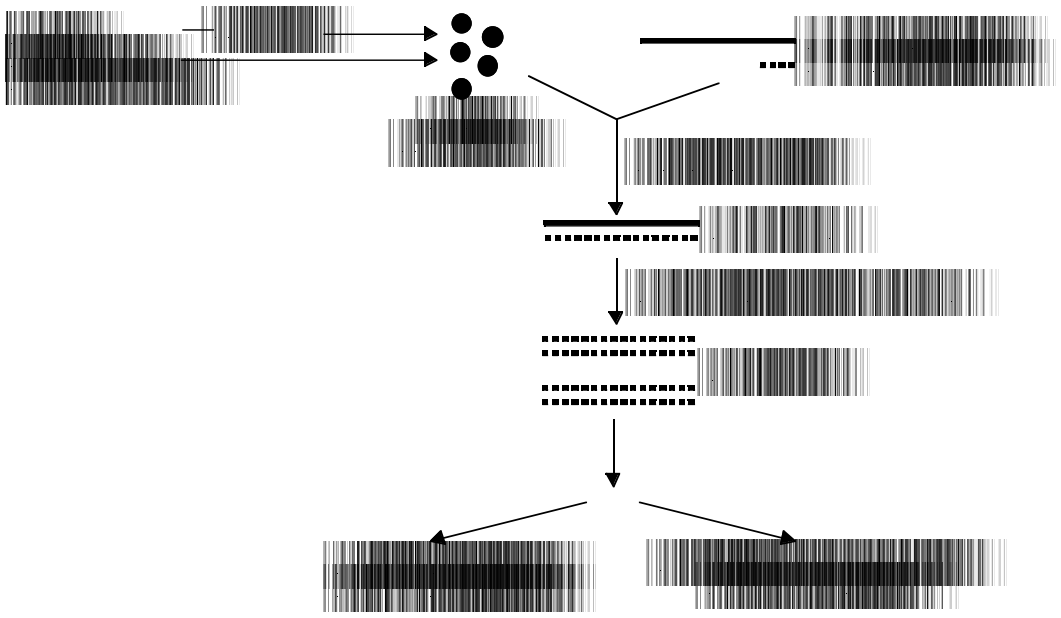

Fig. 1. *Principle of the Amp-RT assay. Detection of RT activity is done by using a known non-retroviral heteropolymeric RNA template and a complementary DNA oligoprimer. RT detection can be done in unprocessed samples or in virus pellets obtained by ultracentrifugation.*

ed reverse transcriptase (RT), an enzyme that transcribes the viral genomic RNA into a double-stranded DNA copy[1]. Assays for detection of RT activity have therefore conventionally been used to identify both known and novel retroviruses. However, their use has been limited to the detection of retroviral particles in culture supernatants since these assays are less sensitive than virus-specific methods such as protein antigen capture or genomic amplification by PCR[2-5].

Recently, several ultrasensitive RT assays such as PERT and Amp-RT have been described[6-9]. All these assays use PCR amplification for the detection of the RT-generated cDNA but differ in the RNA template and testing conditions. The sensitivity of these assays is several orders of magnitude higher than conventional RT assays allowing the detection of RT activity derived from 1-10 HIV-1 particles[7,10]. This article reviews data generated by Amp-RT in three applications: i) Detection of RT activity from HIV-1 variants in plasma, ii) Quantitation of plasma RT in HIV-1 infections and relationship to RNA virus loads, and iii) Screening of phenotypic resistance to HIV-1 RT inhibitors in plasma from HIV-1-infected patients treated with antiretroviral therapy.

Principle of the Amp-RT Assay

Like standard RT assays, Amp-RT detects HIV-1 by measuring the enzymatic activity of HIV-1 RT. Detection of HIV-1 RT activity is done by measuring the ability of a sample to produce a cDNA copy of a known heterologous RNA template by extending a complementary DNA oligoprimer (Fig. 1). The exogenous RNA template that is used is derived from the encephalomyocarditis virus (EMCV) genome and is generated by *in vitro* transcription[9]. The integrity and the purity of the EMCV RNA template are essential for both the sensitivity and the specificity of the Amp-RT assay, since presence of residual EMCV DNA would result in false-positive results or background Amp-RT signals[9].

An important component of the Amp-RT assay is the PCR amplification of the EMCV cDNA. The PCR amplification of EMCV cDNA results in an increase on sensitivity over conventional RT assays of about 10^6-fold[9]. Thus, Amp-RT has been found to detect 10^{-10} units of recombinant HIV-1 RT activity or the RT activity contained in 1-10 HIV-1 particles[10]. Analysis of sucrose gradient fractions of HIV-1 culture supernatants has shown that the peak of detection of RT activity by Amp-RT corresponds to a density of 1.13-1.16 g/mL as expected for HIV-1, indicating that the RT activity measured is virion-associated[11].

Qualitative detection of RT activity by the Amp-RT assay

Since RT is found in all retroviruses, the enzymatic activity of this marker protein may be measured to detect the presence of retroviruses in culture supernatants and biological fluids, and to address the safety concerns associated with biologically derived products. Analysis of culture supernatants by the Amp-RT assay has demonstrated the ability of the assay to detect RT activity from retroviruses representing different genera. Thus, lentiviruses such as HIV-1 (group M and group O), HIV-2, simian immunodeficiency virus (SIV) and caprine arthritis encephalitis virus (CAEV), oncoviruses such as human T cell lymphotropic virus types I

and II (HTLV-I and HTLV-II), gibbon ape leukemia virus (GALV) and simian retroviruses types 1 and 2 (SRV-1 and SRV-2), or spumaviruses such as simian foamy virus type 3 (SFV-3) have all been successfully detected by Amp-RT demonstrating the generic character of the assay, and supporting its use for detecting known or novel retroviruses in biological fluids[9,11-13] (Fig. 2).

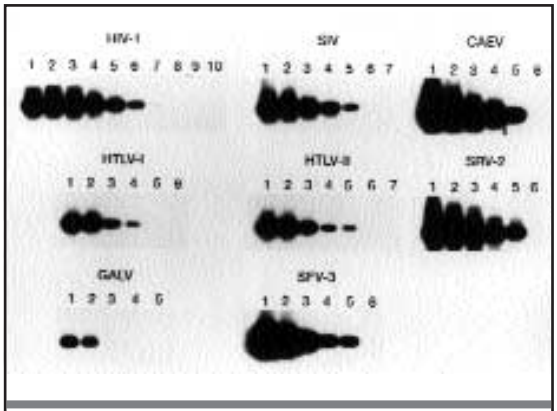

Fig. 2. *Detection of RT activity from several retroviruses by the Amp-RT assay. Lane 1, four µl of original unconcentrated culture supernatant; lanes 2-10, 10-fold serial dilutions (from ref.[9]).*

For rapid screening of retroviral RT activity in plasma, a protocol based on direct analysis of small volumes of unprocessed plasma samples (2 µL) has been developed and validated[10]. Detectable RT activity by Amp-RT has been observed in 93 of 107 samples from HIV-1-infected individuals while none of 118 samples from HIV-1-, HIV-2-, HTLV-I-, and HTLV-II- seronegative blood donors have shown reactivity in the assay. The lack of detectable RT activity observed in some HIV-1-infected patients was associated with a low or undetectable RNA virus load[10]. These results indicated that the Amp-RT assay is a sensitive and specific method that may be used for screening of RT activity from HIV-1 or other retroviruses in plasma samples, providing a rapid tool for analysis of retroviral RT activity in biological fluids.

Quantitative detection of HIV-1 RT activity in plasma of HIV-1 infected persons

Quantitation of HIV-1 plasma virus load provides a useful marker of the overall level of virus replication in the infected host, and therefore is important for studies of pathogenesis, natural history, and efficacy of antiretroviral therapy[14-18]. Quantitation of HIV-1 RNA levels has gained wide acceptance because of its relative technical easy and its usefulness in clinical management of HIV-1-infected patients. To date, several commercially available assays are being used to determine HIV-1 RNA levels including RT-PCR, nucleic acid sequence-based amplification (NASBA), or branched DNA[19-21]. These assays give comparable results and are currently being used in clinical practice for therapeutic management of HIV-1-infected patients to monitor both the efficacy of antiretroviral therapy and disease progression[14,22-24].

Reverse transcriptase is a virion-associated enzyme and therefore, represents an additional virus marker for virus load analysis. Quantitation of plasma virus loads based on levels of RT activity may have three main advantages compared to RNA-based quantitations. First, since functional RT is one of the requirements for virus infectivity and since virus particles that lack active RT enzymes are not infectious, RT-based plasma virus load may represent the pool of plasma virus that is potentially infectious. Second, detection of RT is sequence-independent and is not compromised by sequence variabilities and, therefore, can be used to quantitate virus loads in persons infected with non-subtype B HIV-1. Third, the number of active RT molecules per HIV-1 particle is higher than the number of RNA molecules, thus providing an increased sensitivity over RNA-based quantitations in samples with low virus loads.

Quantitation of plasma virus loads based on levels of functional RT by Amp-RT is done by using an external standard curve of known units of HIV-1 RT activity[10]. The standard used for quantitation can be an HIV-1 isolate with known RT activity per virion or, alternatively, HIV-1 RT with known specific activity. If an HIV-1 isolate is used, the amount of RT activity per virion in that particular virus stock can be easily estimated by comparing the Amp-RT signal obtained with the virus stock with the signal obtained with known units of HIV-1 RT activity. Since Amp-RT is an enzymatic assay, the results are expressed as units of RT activity. Figure 3 shows the dynamic range of Amp-RT, which extends over 4 log[10]. Identical linearities are seen when known HIV-1 virion numbers or known units of recombinant HIV-1 RT are used (Fig. 3). Quantitative detection of RT activity by Amp-RT is done by an ELISA-based non-radioactive oligoprobing system using a digoxigenin-labelled EMCV-specific probe[10].

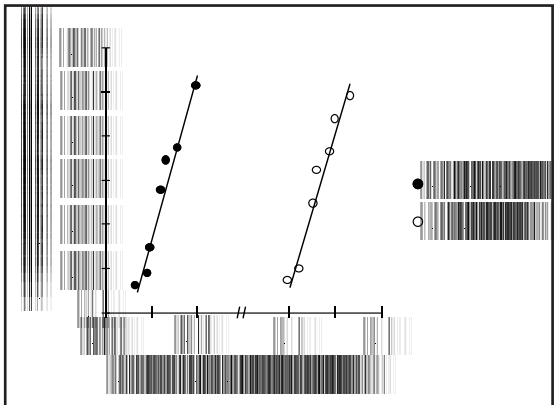

Fig. 3. *Linearity analysis of a quantitative Amp-RT assay by using recombinant HIV-1 RT or HIV-1 virions as standards (from ref.[10]).*

RT detection and quantitation by Amp-RT can be done in culture supernatants or in plasma samples. RT activity in plasma can be directly quantitated in

unprocessed plasma samples or in virus pellets obtained by ultracentrifugation. Direct testing of small volumes of unprocessed plasma samples (2 µL) allows detection of RT activity in samples containing 500-1,000 RNA copies/mL is highly specific, and provides a rapid method for virus load analysis[10]. However ultracentrifugation of plasma samples has several advantages. First, it obviates the need for running an additional Amp-RT reaction needed to correct for possible RT inhibition that may occur when unprocessed plasma samples are used. Second, virus pellets can be concentrated and therefore, larger volumes of plasma (e.g., 10 µL) can be tested by Amp-RT, thus increasing the sensitivity of the assay. Higher sensitivity may be of particular importance for monitoring low levels of plasma viral load such as those seen in patients receiving combination antiretroviral therapy. However, the specificity of the Amp-RT assay using >10 µL of plasma has not been fully validated.

Relationship between RT and RNA virus load

To study the relationship between RT and RNA virus loads, levels of both RT activity and viral RNA have been measured in plasma from HIV-1-infected patients. Patterns and kinetics of plasma viremia measured by Amp-RT were found to be similar to those derived using RNA-based quantitation assays both during the seroconversion period and following treatment with nevirapine[10,25] (Fig. 4). The analysis of the relationship between RT and RNA virus load can be made by determining RT/RNA ratios. These ratios were found to be constant within the same patient during the seroconversion period. However, RT/RNA ratios varied in different individuals during both the early and late stages of HIV-1 infection. The level of variation was higher than that associated with assay variability possibly reflecting variabilities in levels of virion-associated RT. These variabilities may be due to differences in either the amount of mature RT subunits packaged into virions or in levels of particle-associated RT activity[10,26]. The clinical implications of these differences in RT/RNA ratios are not known, and new studies are necessary to examine if RT-based plasma virus loads alone or RT/RNA ratios are better viral markers than RNA in predicting the rate of disease progression and the risk of virus transmission.

Quantitation of plasma virus load in non- subtype B HIV-1 and in HIV-2

Because of its generic nature, the Amp-RT assay can also be used to quantitate plasma virus loads in patients infected with non-subtype B HIV-1 or other retroviruses. For instance, RT-based plasma virus load has been measured in longitudinal samples from two HIV-1 group O-infected treated with RT and protease inhibitors[13]. Since commercially available assays that measure HIV-1 RNA levels in

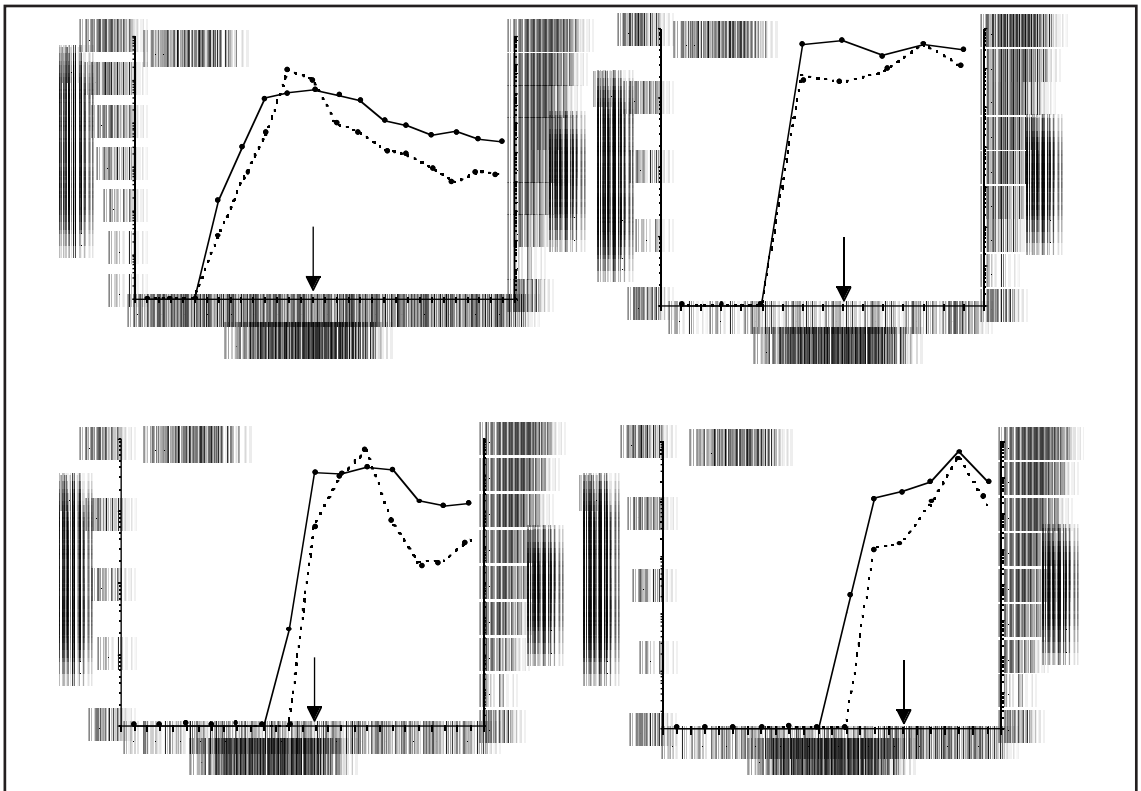

Fig. 4. *RT activity and RNA virus load in 4 HIV-1 seroconversion panels. Dotted line corresponds to levels of RT determined by Amp-RT; solid line corresponds to RNA virus loads determined by RT-PCR. Arrow represents 1st detectable antibody result by HIV-1/2 EIA (modified from ref.[10]).*

plasma are not adequate for quantitation of group O RNA genomes, RT detection by Amp-RT may provide a useful virus marker to derive information on the efficacy of antiretroviral therapy in HIV-1 group O-infected patients[27,28]. In addition to group O infections, the assay has been successfully used to quantitate plasma virus load in other retroviral infections such as HIV-2, thus providing valuable information on levels of virus replication during both the asymptomatic and symptomatic period of HIV-2 infections[12].

Detection of phenotypic resistance to HIV-1 RT inhibitors by direct analysis of RT activity in plasma

The management of HIV-1 disease has significantly improved during the past few years with the use of HIV-1 RT and protease inhibitors. Two classes of HIV-1 RT inhibitors are being currently used for treatment of HIV-1-infected individuals: Nucleoside and non-nucleoside RT inhibitors. The non-nucleoside RT inhibitors (NNRTIs) comprise a series of structurally diverse compounds specific for HIV-1, and do not require metabolic activation to manifest their antiviral effects[29]. In contrast, nucleoside RT inhibitors (NRTIs) share a similar mode of action, are only significantly active following conversion to the triphosphate form, and are not specific of HIV-1[30].

Despite the initial antiretroviral activity of RT inhibitors, the benefit of treatment with these agents is of limited duration mainly due to the emergence of drug-resistant variants of HIV-1. Methods for detecting drug resistance viruses may therefore provide valuable information for treatment decisions and patient management, and their implementation in clinical practice is currently being evaluated[31]. To date, several genotypic and phenotypic tests are being used to monitor the emergence of HIV-1 resistance to RT inhibitors. Genotypic tests are rapid and are widely used to monitor known mutations associated with resistance. Among these, primer-specific PCR, point mutation, and reverse hybridization assays are the most commonly used[18,32-37]. However, clinical monitoring of resistance by genotypic testing may not detect resistance mediated by unrecognized mutations. In addition, genotypic testing cannot detect potential synergistic or antagonistic effects of complex mutation patterns arising from combination therapy with different RT inhibitors. The transient suppression of phenotypic resistance to zidovudine (AZT) conferred by the M184V or the L74V mutation illustrates the effect that combinations of mutations may have in a given phenotype indicating that detection of specific mutations may not always be associated with phenotypic resistance[38-40].

Phenotypic assays provide definitive information on resistance and are well suited to assess complex resistance patterns that may arise from combination. Phenotypic resistance to HIV-1 RT inhibitors has conventionally been measured by RT activity and replication-based assays[41-46]. However, the use of conventional RT assays for drug susceptibility testing in plasma is limited by the inability of these tests to detect low levels of RT activity.

The Amp-RT assay overcomes the lack of sensitivity of conventional RT assays for detection of HIV-1 RT activity in plasma and therefore, provides an additional tool to monitor the development of drug resistance. The basic principle of using Amp-RT as a phenotypic assay is illustrated in figure 5. The RT from highly resistant isolates can generate cDNA copies of the EMCV template in the presence of inhibitor, which can be amplified by PCR. In contrast, RTs from sensitive isolates are completely inhibited resulting in undetectable EMCV PCR product (Fig. 5). Enzymatic resistance of HIV-1 RT to RT inhibitors can be measured by determining Amp-RT IC_{50} values for the drug.

a) *Detection of phenotypic resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs)*

The three NNRTIs currently approved for treatment of HIV-1-infected patients are nevirapine, delavirdine and efavirenz. This class of compounds directly inhibit HIV-1 RT by binding non competitively to conserved residues of the p66 subunit of RT, altering its structure and modifying the active site of the enzyme[29,47,48]. The binding site is a hydrophobic pocket close to the polymerase catalytic site in the p66 subunit of RT, leading to a significant slowing rate of polymerization catalyzed by the enzyme. Since these compounds share a common mechanism of action and bind to a common site of the enzyme, any slight variation due to single point mutations may have a significant impact on the sensitivity to all of them. These point mutations are well characterized and result in the loss of stabilizing interactions and emergence of steric and thermodynamic barriers for NNRTI binding[47].

The effect that nevirapine resistance mutations have on the enzymatic function of HIV-1 RT can be directly measured by determining the level of RT inhibition observed in Amp-RT reactions done in the presence of nevirapine[25]. Drug susceptibility results determined by Amp-RT in several WT and nevirapine-resistant HIV-1 isolates correlate with IC_{50} values determined by conventional phenotypic assays[25] (Fig. 6). Furthermore, phenotypic resistance to nevirapine was detected in RTs carrying several nevirapine resistance mutations including Y181C, V106A, K103N, Y190A, G190A, and Y188L, indicating that the Amp-RT assay can successfully detect resistance mediated by a wide variety of mutations in the HIV-1 RT[25,49-51].

A simple testing strategy using a single nevirapine concentration in the Amp-RT assay (50 μM) was also found to be useful for rapid screening of nevirapine resistance in plasma samples. The decrease in the RT inhibition values observed overtime in patients treated with nevirapine correlated with an increase in the level of detectable Y181C mutation and was associated with an increase in the Amp-RT

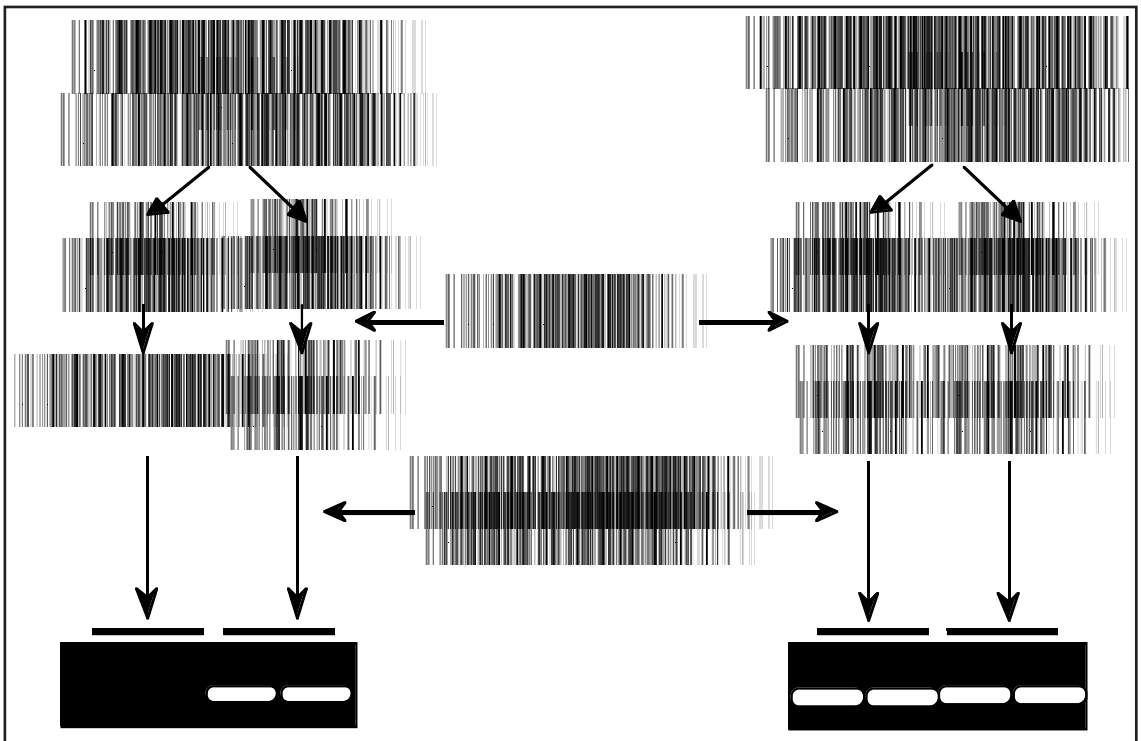

Fig. 5. *Principle of the Amp-RT-based phenotypic assay for detection of resistance to RT inhibitors. Susceptibility of RT is determined based on the level of inhibition produced by drug, and is measured by running quantitative Amp-RT reactions in the presence and absence of the relevant inhibitor.*

		IC_{50} (uM)/fold*	
HIV-1 isolate	*Mutations*	*Culture*	*Amp-RT*
X267-1	-	0.04	4
X165-6	-	0.07/1.7	11/2.7
X82-5	K103N, Y181C	4.6/115	491/123
X403-4	Y181C	9/225	571/143
W786-6	K103N, Y181C	20/400	2283/570
X165-11	G190A, Y181C	22/550	1474/369
S469-2/M3	Y188L	100/2500	2642/661
V818-5	G190A, Y181C	>100/>2500	9804/2451

* fold-resistance compared to isolate X267-1

Fig. 6. *Correlation between nevirapine susceptibility determined by the Amp-RT-based phenotypic assay and drug susceptibility results obtained by a plaque-reduction assay. Amp-RT IC_{50} values from two reference sensitive (X267-1 and X165-6) and six nevirapine-resistant HIV-1 isolates (X82-5, X403-4, W786-6, X165-11, S469-2/M3, and V818-5) are shown along relevant genotype and IC_{50} values for nevirapine derived by plaque-reduction assay (adapted from ref.[25]).*

IC_{50} value for nevirapine[25] (Fig. 7). All these results validated the use of this rapid approach to monitor the development of nevirapine resistance, and suggest that similar Amp-RT-based assays could be used for other NNRTI. The usefulness of this Amp-RT approach for detection of resistance to efavirenz is currently being validated using HIV-1 isolates carrying different mutations associated with resistance.

b) *Detection of phenotypic resistance to nucleoside reverse transcriptase inhibitors (NRTIs)*

NRTIs are activated intracellularly by phosphorylation to the 5'-triphosphate form[30]. The triphosphate form of NRTIs inhibit HIV-1 reverse transcription through competitive inhibition with the native dNTP for RT and chain termination of elongating DNA[30]. Analysis of phenotypic resistance to NRTIs by Amp-RT, therefore, requires the triphosphate form of the relevant inhibitor (e.g. 3TC-TP for 3TC, ddA-TP for ddI, ddC-TP for ddC, or AZT-TP for AZT).

Of the six currently approved nucleoside analogs, 3TC has potent anti-HIV-1 activity and minimal toxicity and is one of the most commonly used drug in combination therapy as first-line treatment for HIV-1-infected patients[52-53]. The high level of phenotypic resistance to 3TC conferred by the M184V mutation results in the lack of significant RT inhibition by 3TC-TP with drug concentrations that completely inhibit wild type (WT) RTs. We developed a screening strategy for 3TC resistance that uses a single concentration of 3TC-TP and demonstrated its ability to differentiate between 3TC-sensitive and resistant RTs[54]. In addition to resistance to 3TC mediated by the M184V mutation, this assay was also able to detect low level cross-resistance to 3TC mediated by mutations conferring multiple dideoxinucleoside resistance (Q151M complex), thus confirming previous findings observed by culture-based assays[55] (Fig. 8).

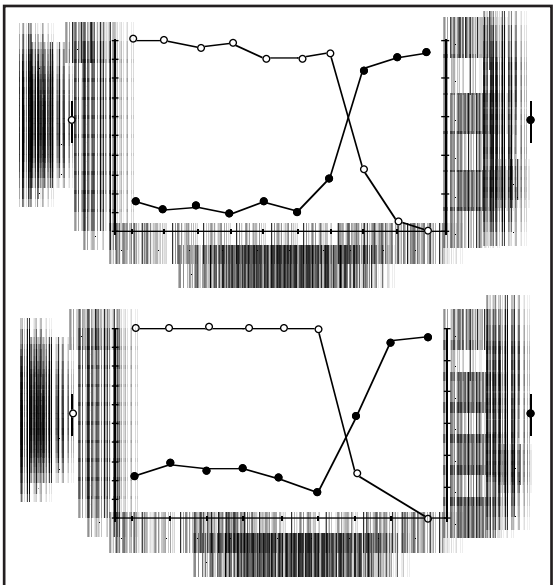

Fig. 7. *Detection of phenotypic resistance to nevirapine in plasma from two HIV-1-infected patients treated with nevirapine monotherapy, and correlation with detection of the Y181C mutation by differential hybridization. Open circles correspond to the RT inhibition values determined by the Amp-RT-based phenotypic assay, and closed circles correspond to the log_{10} ratio between mutant genotypes (Y181C) and a highly conserved region of the HIV-1 RT (MUT/GNR). (adapted from ref.[25]).*

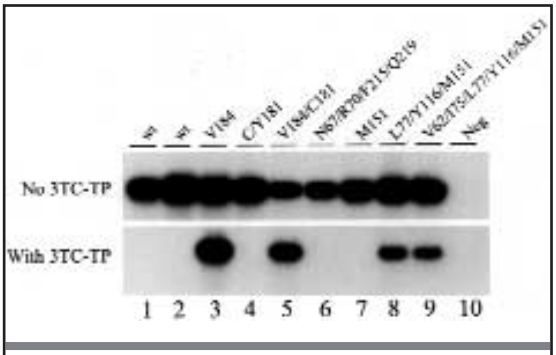

Fig. 8. *Detection of phenotypic resistance to 3TC by analysis of RT inhibition by 5 μM 3TC-TP in the Amp-RT assay. Amp-RT reactions done in the presence and absence of 3TC-TP are shown. Lanes 1 and 2, wild type (wt) HIV-1 RT; Lane 3, 3TC-resistant (M184V); Lane 4, nevirapine-resistant (181C/Y); lane 5, 3TC/nevirapine-resistant (M184V/Y181C); Lane 6, AZT-resistant (D67N/K70R/T215F/K219Q); Lanes 7, 8, and 9, HIV-1 RT carrying different multidrug-resistant mutations; Neg, uninfected culture supernatant.*

The use of a single drug concentration to differentiate between sensitive and 3TC-resistant RTs provides a rapid tool for screening of phenotypic resistance to 3TC. Phenotypic resistance to 3TC has been successfully detected in plasma samples from patients that had evidence of genotypic markers of resistance to 3TC[54]. For other $NRTI_S$ such as ddI and ddC, however, this Amp-RT testing approach using a single concentration of ddA-TP and ddC-TP may not be sufficient to monitor the low level resistance conferred by the ddI or ddC resistance mutations. In this situation, $NRTI_S$Amp-RT IC50 determination is required to provide information on the levels of resistance to these drugs [unpublished observations].

The mechanisms of AZT resistance are not completely understood and affect the use of the Amp-RT assay to measure AZT resistance. Several reports have shown that RTs carrying the classical AZT resistance mutations (e.g., M41L, T215Y/F, K70R or D67N) have similar *Ki* values or IC_{50} values for AZT-TP that wild type RTs[30]. The inability of detecting AZT-resistance by RT assays has also been observed in Amp-RT reactions done with RTs carrying several AZT resistance mutations. However, the assay has been successfully used for screening of phenotypic resistance to AZT mediated by mutations at codon 151 and other positions associated with resistance to multiple dideoxynucleoside analogs, thus providing a rapid tool for surveillance of multidrug resistant phenotypes[56].

Advantages of drug susceptibility testing by Amp-RT

RT-based drug susceptibility testing by Amp-RT has two main advantages compared to replication-based assays. First, conventional phenotypic assays are based on generating virus isolates by standard isolation or by the recombinant virus techniques and are, therefore, labor intensive, costly, and unsuitable for rapid clinical monitoring of drug resistance. In contrast, drug susceptibility testing by Amp-RT does not require virus isolation by culture and provides rapid information on resistance (1-2 days). Second, the Amp-RT assay directly measures phenotypic resistance in HIV-1 from plasma samples, and therefore, does not have selection bias associated with virus isolation in culture[57,58].

The screening assay described above using single concentrations of nevirapine or 3TC-TP can successfully detect phenotypic resistance in mixtures containing as low as 10% resistant viruses in a background of wild type HIV-1. The detection of low proportion of resistant viruses is unique among phenotypic assays and may allow an early detection of resistant viruses in patients receiving these drugs. Detection of resistance in these patients could be used as a marker of treatment failure, therefore providing an opportunity for an early treatment change. Mixtures of wild type and resistant viruses may also be seen in recent seroconverters who have been infected with resistant viruses. In the absence of treatment, the proportion of resistant viruses in these patients can decrease over time. The ability to detect low proportions of resistant viruses makes Amp-RT a useful assay for surveillance studies of drug-resistant viruses in recent seroconverters.

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