A novel TaqMan real-time PCR assay to estimate ex vivo human immunodeficiency virus type 1 fitness in the era of multi-target (pol and env) antiretroviral therapy


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INTRODUCTION

Numerous studies have addressed human immunodeficiency virus type 1 (HIV-1) fitness and the potential effects on virus load, drug resistance to protease (PR) and/or reverse transcriptase (RT) inhibitors (PI and RTI, respectively) and HIV pathogenesis (reviewed by Nijhuis et al., 2001; Quiñones-Mateu & Arts, 2001, 2002). More recent studies have correlated the impairment on virus fitness of PI-resistant variants with an atypical response to antiretroviral therapy, perhaps with consequences on HIV-1 control and disease progression (Deeks et al., 2000, 2001; Barbour et al., 2002). However, it is still unknown whether or not in vitro systems resemble the actual virus fitness in vivo. Most in vitro HIV-1 fitness studies have been performed with recombinant viruses containing HIV-1 genes (e.g. pol), often amplified by PCR directly from patient plasma or cells (Shi & Mellors, 1997; Hertogs et al., 1998; Martinez-Picado et al., 1999; Robinson et al., 2000; Bleiber et al., 2001). One of the main advantages of these recombinant viruses, as opposed to HIV-1 primary isolates, is the ability to determine the impact of specific sites or sequences within a neutral HIV-1 backbone. However, since other genomic regions in the recombinant virus external to those derived from a clinical specimen could have an equal or greater impact on fitness; the in vivo relevance of fitness studies using recombinant viruses is still the subject of debate.

Multiple methods have been used to measure HIV-1 fitness in vitro (Clavel et al., 2000; Nijhuis et al., 2001; Quiñones-Mateu & Arts, 2001), yet few studies have directly compared different methods to measure HIV-1 fitness (Grant et al., 2001; Prado et al., 2002). These studies have suggested that the highly impaired replication capacity of drug-resistant HIV-1 pol mutants may allow the use of recombinant
viruses to estimate overall HIV-1 fitness and its relationship to drug resistance. However, small differences in virus fitness have been more apparent using growth competition experiments than single-cycle assays (Bleiber et al., 2001; Prado et al., 2002). A recent study comparing the individual contribution of PR- and RT-encoding regions to fitness in drug-resistant HIV-1 primary isolates suggested that other genomic regions outside of drug-targeted genes may compensate for the drug-resistant substitutions in pol (Bleiber et al., 2001). In addition, new antiretroviral drugs are being developed that target other regions of the viral genome, which may not be included in these recombinant pol constructions. For example, HIV entry inhibitors that involve viral envelope glycoproteins and their cellular receptors are now under clinical evaluation (Moore & Stevenson, 2000). Therefore, the use of HIV-1 clinical isolates instead of recombinant viruses may help elucidate the interdependence between different viral genes, their effect on virus fitness (i.e. virulence, transmission and pathogenicity) (Quinones-Mateu et al., 2000), multidrug resistance to PI and RTI (Brenner et al., 2002; Quinones-Mateu et al., 2002) and potential resistance to multi-target antiretroviral therapy (e.g. directed against pol and env).

In the absence of a consensus method for quantifying virus replication capacity, many studies have employed different techniques to assess HIV-1 fitness (Quinones-Mateu & Arts, 2001). New technologies such as real-time PCR have also been applied to dual virus detection. de Ronde et al. (2001) used real-time nucleic acid sequence-based amplification PCR and molecular beacons to quantify individual mutant viruses in a mixture. Lu & Kuritzkes (2001) developed a recombinant marker virus assay to perform growth competition experiments between two RT recombinant viruses which were quantified using real-time PCR for the corresponding marker. Several studies have described the use of TaqMan real-time PCR as a sensitive technique to measure and quantify plasma HIV-1 RNA or proviral HIV-1 DNA (Lewin et al., 1999; de Baar et al., 2001; Desire et al., 2001; Hance et al., 2001; Zhao et al., 2002). However, TaqMan real-time PCR has not been used to detect two different HIV-1 primary isolates in growth competition experiments in order to estimate virus fitness.

It is important to note that the term ‘virus fitness’ is commonly employed in both in vitro studies and the clinical setting (Nijhuis et al., 2001; Quinones-Mateu & Arts, 2001). For example, most studies describing the diminished replicative capacity of HIV-1 drug-resistant strains have referred to this phenotype as virus fitness. However, in vivo virus fitness depends on multiple virus and host factors (Quinones-Mateu & Arts, 2001), while ‘replication capacity’ is the intrinsic capacity of the virus to replicate efficiently in an ideal environment (i.e. cell culture) (Nijhuis et al., 2001). Therefore, and in order to distinguish between both approaches, we have denoted ex vivo ‘virus fitness’ to those values estimated using HIV-1 isolates in growth competition experiments to differentiate them from ‘replication capacity’ (in vitro virus fitness) values obtained using pol recombinant viruses. In the present study, we have developed a rapid method for quantifying two different proviral HIV-1 genomes in a mixture using TaqMan real-time PCR, which specifically differentiates subtype B HIV-1 isolates from subtype A or CRF01 AE HIV-1 strains. This method, comprising independent growth competition experiments between a subtype B HIV-1 isolate and two non-subtype B viruses followed by the TaqMan real-time PCR assay, was sensitive, reproducible and allowed a wide dynamic range of detection to measure HIV-1 fitness. We compared our new assay with a single-cycle replication capacity assay to quantify the reduction in fitness of drug-resistant recombinant viruses, demonstrating consistent results between both methods.

**METHODS**

**Viruses.** All virus stocks (i.e. primary isolates and recombinant viruses) were propagated and expanded in PHA-stimulated, IL-2-treated PBMCs, as described previously (Quinones-Mateu et al., 2000). Four syncytium-inducing HIV-1 isolates (laboratory-adapted strain HIV-1B-HXB2 and three primary isolates HIV-1 A-92UG029, HIV-1B-92US076 and HIV-1AE-CM196) were obtained from the AIDS Research and Reference Reagent Program. TCD50 titres were determined for each isolate in triplicate with serially diluted supernatants of each virus propagation. RT activity (Torre et al., 2000) in culture supernatants on day 8 of culture was used to calculate TCD50 values using the Reed and Muench method (Reed & Muench, 1938). Titres are expressed as IU ml−1.

**Growth competition experiments to estimate HIV-1 fitness.** All dual infection/competition experiments were carried out as described previously (Quinones-Mateu et al., 2000, 2002) with minor modifications. Briefly, each subtype B HIV-1 primary isolate or recombinant virus was competed against two different non-subtype B HIV-1 control strains (HIV-1A-92UG029 and HIV-1AE-CM196) in a 1:1 initial proportion using a m.o.i. of 0-01 IU per cell (Fig. 1A). Of these virus mixtures, 1 ml was incubated with 1 × 10⁶ PBMCs for 2 h at 37 °C and 5% CO₂. Subsequently, the cells were washed three times with 1× PBS and then resuspended in culture medium (1 × 10⁶ ml⁻¹). Cells were washed and fed with medium after 4 days. Supernatants and cells were harvested at day 8 and stored at −80 °C for subsequent analysis.

**TaqMan real-time PCR assay for detection of two HIV-1 strains in a mixture.** Before quantifying both HIV-1 variants in the growth competition, the initial amount of cellular and proviral HIV-1 DNA in each TaqMan real-time PCR was first determined. Albumin DNA was quantified to determine the input level of cellular DNA in the sample and was used as an endogenous reference to normalize variations due to differences in the PBMC count or DNA extraction. Primers Alb-S (5′-GCTGTCTACCTCCTGTGCGGTCTG-3′) and Alb-AS (5′-AAACTCATGGGAGCTGCTGGTT-3′) were used to quantify the human albumin gene (Laurendeau et al., 1999). A 199 bp pol gene fragment corresponding to proviral HIV-1 DNA from both HIV-1 strains in the mixture was amplified by PCR using universal primers P1-S (5′-TGGGATGGTACCCAGACCA-3′, nt position 4147 corresponding to the HIV-1HXB2 isolate; http://hiv-web.lanl.gov) and P2-AS (5′-CTGGCTACTATTTCTTTTGCTA-3′, nt position 4324) (Yerly et al., 1992). The 25 μl PCR mixture for albumin or HIV-1 DNA amplification consisted of 5 μl (1/40 of the DNA extract), primers Alb-S and Alb-AS or P1-S and P2-AS (0-2 μM each), 0-2 mM dNTPs, 5 mM MgCl₂, 1-25 U Platinum
**A. Growth competition experiment**

![Diagram of growth competition experiment](http://vir.sgmjournals.org)

**B. Real time PCR / TaqMan strategy**

![Diagram of real time PCR / TaqMan strategy](http://vir.sgmjournals.org)

**C. Construction of recombinant viruses**

![Diagram of construction of recombinant viruses](http://vir.sgmjournals.org)

Fig. 1. Schematic representation of growth competition experiments, TaqMan real-time PCR detection and generation of HIV-1 recombinant viruses. (A) Individual dual infections with a subtype B query HIV-1 isolate and each one of a control strain (HIV-1A-92UG029 or HIV-1AE-CMU06) were performed at an m.o.i. of 0-01 IU per cell. Wells I and III correspond to positive control for the query and control viruses, respectively. (B) Three subtype-specific primer/probe sets were designed to quantify the proportion of both HIV-1 variants in the dual infection. Subtype B-specific primers (Bgag-S/Bgag-AS3) and probe (pBgag-ROX) amplify and recognize a conserved region within the subtype B HIV-1 gag gene. Similarly, subtype A-specific primers (A2env-S/A2env-AS2) plus probe (pA2envFAM) and CRF01_AE-specific primers (E1env-S/E1env-AS) and probe (pE1envFAM) amplify and recognize conserved regions in the HIV-1 env genes of clade A and the circulating recombinant form CRF01_AE, respectively. (C) PCR fragments derived from patient plasma or PBMC samples were used to construct three different HIV-1 recombinant viruses (see Methods for details).

To quantify the proportion of both HIV-1 variants in the mixture, three sets of subtype-specific primers and probes were designed (Fig. 1B). Subtype B-specific primers, Bgag-S (5’-GGAGCTAGAACATTACCCAGC-3’, nt position 906), Bgag-AS3 (5’-TTATGACTTACAAACGCTG-3’, nt position 1104) and probe pgag-ROX (5’-ROX-TACTGGGCAGCAATGGACCT-3’, nt position 968), where 6-carboxy-X-rhodamine (ROX) is the reporter fluorochrome and black hole quencher (BHQ) corresponds to the quencher. This primer/probe combination was designed to recognize a conserved region within the subtype B HIV-1 gag gene. In addition, two sets of primers and probes were designed to recognize conserved regions in the HIV-1 env genes of clade A and the circulating recombinant form CRF01_AE. Subtype A-specific primers were A2env-S (5’-CTATATGTTACCAACATTACACAGC-3’, nt position 7073), A2env-AS2 (5’-ATATGACTTACAAACGCTG-3’, nt position 7338) and probe pA2envFAM (5’-FAM-CAGAAGAAGGGTACATAGGAGACCC-3’, nt position 7133), CRF01_AE-specific primers were E1env-S (5’-TTATGACTTACAAACGCTG-3’, nt position 7144), E1env-AS2 (5’-CTATATGTTACCAACATTACACAGC-3’, nt position 7403) and probe pE1envFAM (5’-FAM-CGGGAGGCGTCTATA-3’, nt position 7318). FAM and TAMRA correspond to the 6-carboxyfluorescein reporter fluorochrome and the 6-carboxytetramethylrhodamine quencher, respectively.

These three sets of primer/probe combination allowed subtype-specific PCR amplification and hybridization so that cross-hybridization between subtype B probes and subtypes A or AE did not occur. PCR products corresponding to three primary isolates (i.e. HIV-1B-92UG029, HIV-1A-92UG029 and HIV-1AE-CMU06) were used to verify the sensitivity and specificity of these oligonucleotides. Each 25 μL TaqMan real-time PCR mixture contained 10 μL DNA (usually 1/20 of the DNA extract), 1 μL Taq buffer (Invitrogen), 6 mM MgCl₂, 0-2 mM dNTPs, 0-2 μM of each primer, 0-4 μM of probe and 1-25 U Platinum Taq DNA polymerase (Invitrogen). PCR conditions consisted in one cycle of denaturation (95°C for 10 min), followed by 40 cycles of amplification. Each PCR experiment was performed in triplicate to ensure reproducibility. 

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**Taq DNA polymerase (Invitrogen), 1x PCR buffer (Invitrogen) and SYBR Green dye diluted 1:2500 (Sigma).** PCR conditions for both albumin and HIV-1 DNA amplification have been described previously (Desire et al., 2001). Standard curves were constructed by cloning one copy of the PCR product corresponding to the human albumin into the pCR-II-TOPO vector (Invitrogen) (i.e. pAlbumin) or using pNL4-3, the infectious HIV-1 molecular clone (obtained through the AIDS Research and Reference Reagent Program). Concentration of these plasmids was determined by spectrometry at 260 nm. Single-stock solutions of serial dilutions from 10⁷ (albumin) or 10⁶ (HIV-1) to 10 copies were prepared and stored at -20°C.

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**http://vir.sgmjournals.org**
Table 1. Virological parameters of APV-selected HIV-1 mutants

<table>
<thead>
<tr>
<th>Virus*</th>
<th>PR mutations†</th>
<th>RT mutations†</th>
<th>APV susceptibility (fold change in IC_{50})‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>10F + Gag 449I</td>
<td>None</td>
<td>1.8</td>
</tr>
<tr>
<td>M2</td>
<td>10F, 84V + Gag 449I</td>
<td>None</td>
<td>8.4</td>
</tr>
<tr>
<td>M4</td>
<td>10F, 46I, 50V + Gag 449I</td>
<td>None</td>
<td>21</td>
</tr>
<tr>
<td>M5</td>
<td>10F, 46I, 47V, 50V + Gag 449I</td>
<td>None</td>
<td>48</td>
</tr>
</tbody>
</table>

*APV-selected HIV-1 mutants were obtained from culture supernatant fluids as part of a previous study (Prado et al., 2002).
†Drug resistance-associated mutations, as described previously (http://www.iasusa.org). PR, Protease; RT, reverse transcriptase; none, wild-type sequence as compared to HIV-1HXB2 (http://hiv-web.lanl.gov). Primary mutations are indicated in boldface letters.
‡In vitro-selected HIV-1 mutants were assayed for susceptibility to APV. In this assay, drug susceptibility is considered to be reduced when there is a 2.5-fold increase in IC_{50} with respect to the reference strain.

by 40 cycles of amplification (95 °C for 15 s, 63 °C for 1 min and 66 °C for 1 min). All real-time PCR amplifications, data acquisition and analysis were performed using the Smart Cycler System, software version 1.2d (Cepheid).

HIV-1 isolates used to test the TaqMan real-time PCR assay.
To test our method to detect two different HIV-1 strains in a mixture, HIV-1 isolates were obtained from two different sources. First, four HIV-1 strains with different levels of susceptibility to the PI amprenavir (APV) (i.e., collected during serial passages of HIV-1_HXB2 in the presence of increasing concentrations of the drug) were obtained from a previous study (Table 1) (Prado et al., 2002).
Second, HIV-1 primary isolates with distinct patterns of drug resistance mutations in pol were obtained from three different HIV-1-infected individuals treated at the Hospital Universitari Germans Trias I Pujol in Badalona, Spain (Table 2) (Cabanà et al., 1999).

RT-PCR, PCR and sequencing analysis of HIV-1 pol. Viral RNA was purified from pelleted virus particles (from cell-free plasma or culture supernatant centrifuged at 32 000 g for 40 min) using QIAamp Viral RNA Mini kit (Qiagen), then reverse-transcribed using the GeneAmp Gold RNA PCR kit (PE Biosystems) and the corresponding antisense external primer. Viral cDNA was amplified further by PCR using a set of external and nested primers described below. Both external and nested PCR reactions were carried out in a 100 μl reaction mixture with defined cycling conditions (Quiniones-Mateu et al., 2000). For all growth competition experiments, proviral DNA was extracted from lysed PBMCs using the QIAamp DNA Blood kit (Qiagen), then amplified by PCR using a different set of external and nested primers, depending on the method used to detect both HIV-1 strains in the mixture (i.e., TaqMan real-time PCR or heteroduplex-tracking analysis (HTA), see below) (Quiniones-Mateu et al., 2002). PCR products were purified using the QiAquick PCR Purification kit (Qiagen). Finally, the full PR-encoding region and the first 296 aa from the RT polymerase domain (from viruses listed in Tables 1 and 2) were sequenced using AP Biotech DYEnamic ET Terminator cycle with Thermosequenase II (Davis Sequencing LCC). Nucleotide sequences were edited and translated using BIOEDIT, version 5.07 (Hall, 1999). Multiple alignment and sequence analyses were performed using CLUSTAL X, version 1.8 (Thompson et al., 1997).

HTA for detection of two HIV-1 env fragments in a mixture.
To evaluate the new TaqMan real-time PCR assay, nested PCR products (C2-C4 of env) from selected growth competition experiments were analysed using HTA, as described previously (Quiniones-Mateu et al., 2000, 2002; Ball et al., 2003).

Construction of PR and RT HIV-1 recombinant viruses. To analyse the contribution of different HIV-1 genomic regions to virus fitness, different HIV-1 recombinant viruses were constructed (Fig. 1C). PR and RT recombinant viruses were prepared as described previously (Gutierrez-Rivas et al., 1999; Mas et al., 2000).
Briefly, proviral DNA was extracted from lysed PBMCs from three different patients with different susceptibility to PI and RTI (i.e. longitudinal samples P96 and P98 and two cross-sectional samples, J94 and A94) (Table 2) (Cabanà et al., 1999). Full-length PR- or RT-encoding sequences were amplified by nested PCR. PCR products were then co-transfected with a PR-deleted (pHIVProBstEII) (Maschera et al., 1995) or an RT-deleted (pHIVRTBstEII) (Kellam & Larder, 1994) HIV-1 HXB2-based clone into SupT1 cells, as described previously (Gutierrez-Rivas et al., 1999). Culture supernatants were harvested when the HIV-1 p24 antigen concentration surpassed 20 ng ml⁻¹. The complete PR-encoding sequence and the first 750 nt of the RT-encoding sequence of the progeny viruses were determined and compared with the genotype of the initial PCR products.

Estimation of drug susceptibility and virus replication capacity.
A rapid recombinant assay was used to measure the drug susceptibility and replication capacity of recombinant viruses (PhenoSense HIV) (Petropoulos et al., 2000; Prado et al., 2002).
Briefly, this assay involved the use of a replication-incompetent HIV-1 vector in which a luciferase gene has been inserted into a deleted portion of env (Petropoulos et al., 2000). A PCR fragment derived from patient plasma samples or virus stocks (containing the 3′ end of gag and both PR- and RT-encoding regions) (Fig. 1C) was introduced into this vector and co-transfected with a murine leukaemia virus env gene construction. HIV-1 3′Gag/PR/RT vectors were prepared as libraries to capture most of the viral sequence pool heterogeneity contained in the patient. The susceptibility (IC_{50} values) of these recombinant vectors to a panel of PI and RTI was compared to a reference vector containing the PR and RT sequences derived from HIV-1_{NL4-3}. In addition, the relative replication capacity of these viruses was determined by normalizing the luciferase activity produced at 72 h post-infection in the absence of drug, with the luciferase activity expressed by cells infected with the parental HIV-1_{NL4-3} strain to give relative light units. Replication capacity measurements were expressed as a percent of the mean of a large wild-type HIV population.
was determined by HTA and/or TaqMan real-time PCR assay and compared to production in monoinfections, as described previously (Quiñones-Mateu et al., 2000, 2002). Briefly, a relative fitness ($w$) value for each virus in the competition was estimated by the production of each individual HIV-1 strain in the dual infection. Total relative fitness was calculated as the average of the two relative fitness values, corresponding to the competition between each subtype B HIV-1 isolate or recombinant virus and each one of the non-subtype B HIV-1 control strains. The total relative fitness was then compared and expressed as a percentage of a wild-type subtype B HIV-1 primary isolate (HIV-1B-92US076, taken as 100%) (Quiñones-Mateu et al., 2002).

**Statistics methods.** Pearson product moment correlation coefficient was used to determine the strength of association or correlation between different methods or techniques to measure virus fitness. All statistical tests were performed using SIGMASTAT, version 2.03 (SPSS).

**RESULTS**

Detection and quantification of two HIV-1 strains in a dual infection by a TaqMan real-time PCR assay

To optimize the conditions of our new growth competition/TaqMan assay to estimate HIV-1 fitness, serial dilutions of two different plasmid constructions with copy numbers ranging from 1 to $10^7$ copies per reaction were analysed in triplicate. First, we tested the ability to quantify and normalize the input level of cellular DNA and proviral HIV-1 DNA from each growth competition experiment using real-time PCR and SYBR Green dye. Fig. 2(A) shows both real-time PCR results and standard calibration curves using human albumin and HIV-1 pol PCR products (from plasmids pAlbumin and pNL4-3, respectively). Down to 10 copies per reaction, all single dilutions, were positive, showing a dynamic range of at least six orders of magnitude, with strong negative correlations between the initial number of target copies in the PCR and the PCR cycle threshold ($r = -0.98$, $P = 0.04$, for both albumin and proviral HIV-1 DNA; Pearson product moment). Therefore, subsequent quantifications of two HIV-1 strains in each growth competition experiment were normalized using these two parameters.

Further experiments evaluated the ability of three different subtype-specific HIV-1 primer/probe sets (i.e. subtype B gag and subtype A- or CRF01_AE env oligonucleotides) to discern and quantify specific HIV-1 strains in a mixture. Different concentrations of HIV-1B-NL4-3, HIV-1A-92UG029 and HIV-1AE-CMU06 were mixed in vitro and amplified by PCR using subtype-specific primer/probe sets and TaqMan real-time PCR. All three primer/probe sets were able to specifically PCR amplify and quantify the corresponding HIV-1 strain from a mixture with two other HIV-1 subtypes (Fig. 2B). Similar to the SYBR Green dye detection, PCR results using subtype-specific TaqMan probes showed a strong negative correlation between HIV-1 input and the PCR cycle threshold ($r = -0.99$, $P = 0.03$; $r = -0.97$, $P = 0.04$; and $r = -0.99$, $P = 0.02$ for subtypes B, A and AE.
A. Quantifying cellular and proviral DNA input

**ALBUMIN**

**HIV-1pNL4-3**

Real time PCR results

Calibration curves

B. Subtype-specific HIV-1 quantification

Subtype B in the presence of subtype A and AE viruses

Subtype A in the presence of subtype B virus

Subtype AE in the presence of subtype B virus

Fig. 2. Optimization of the conditions to use TaqMan real-time PCR for detection of two HIV-1 strains in a mixture. (A) Quantification of cellular (albumin) and proviral HIV-1 DNA (199 bp pol gene PCR fragment) were used to normalize the initial input of template for further subtype-specific PCR reactions. (B) Viruses from different subtypes were mixed in order to evaluate the ability of three subtype-specific primer/probe sets (see legend of Fig. 1) to discriminate and quantify two HIV-1 isolates in a mixture. Real-time PCR results and calibration curves are shown when increasing amounts of: (i) a subtype B HIV-1 isolate (B-NL4-3) were quantified in the presence of fixed amounts of two other HIV-1 isolates (subtype A, HIV-1_A92UG029; CRF01_AE, HIV-1_AE-CMU06); (ii) a subtype A HIV-1 isolate (A-92UG029) in the presence of subtype B (B-NL4-3); and (iii) a CRF01_AE HIV-1 isolate (AE-CMU06) in the presence of subtype B (B-NL4-3). Linear dynamic ranges and regression values are indicated for each subtype-specific primer/probe set.

HIV-1 competitions and estimation of virus fitness: comparison between HTA and TaqMan real-time PCR assays

We have described previously the use of HTA to identify two HIV-1 isolates in a mixture and estimate their virus fitness (Quiñones-Mateu et al., 2000, 2002; Ball et al., 2003). In this study, we have refined our dual competition assay.
by using TaqMan real-time PCR assay to accurately detect and measure two HIV-1 isolates in a growth competition experiment. However, several controls were needed to validate this new assay as an accurate method to quantify virus production and fitness. Four different competitions were established between two subtype B HIV-1 strains (i.e. HIV-1HXB2 and HIV-192US076) and the two non-subtype B HIV-1 primary isolates used as controls in our assay (i.e. HIV-1A-92UG029 and HIV-1AE-CMU06, see Methods). Detection and quantification of virus production was performed by HTA and TaqMan real-time PCR assay (Fig. 3). In this case, both subtype B viruses outcompeted the non-B control isolates. Both techniques, HTA and TaqMan, were able to distinguish clearly between subtype B and non-clade B virus production. However, in all cases, the TaqMan assay was more sensitive than HTA and detected smaller amounts of HIV-1 control isolates (Fig. 3). This increase in the sensitivity to detect low levels of virus production in a mixture using TaqMan real-time PCR allowed a better quantification and estimation of HIV-1 fitness. Therefore, all subsequent competition experiments were analysed using the TaqMan method of detection.

**Ex vivo virus fitness and virus replication capacity of APV-resistant HIV-1 strains**

To further test this new method to estimate HIV-1 fitness, we first analysed the *ex vivo* virus fitness of four HIV-1 strains with different levels of susceptibility to APV (Table 1) and compared these results with virus replication capacity values obtained previously using a modified version of the PhenoSense drug-susceptibility assay (Prado *et al.*, 2002). Replication kinetics analyses of these APV-resistant strains showed similar results when either RT activity or production of Gag p24 antigen were measured in parallel cultures (Fig. 4A). Both assays (RT and p24...
measurements) showed a marked reduction in replication of the M5 virus, corresponding with the accumulation of APV-resistance mutations in the PR gene and a reduction in the susceptibility to this PI (Table 1 and Fig. 4A). However, when more sensitive assays were used (i.e. growth competition experiments and single-cycle replication capacity assay in the absence of drug), significant differences between the fitness of the distinct APV-selected viruses were observed (Fig. 4B). Most virus isolates and their 3′Gag/PR/RT recombinant viruses showed a progressive reduction in virus fitness and replication capacity when compared with the wild-type reference strain (i.e. HIV-1-B92US076 and HIV-1NL4-3, respectively). Overall, virus M2, which contains the PR mutations 10F and 84V and the 449F mutation in gag, showed the most impaired virus fitness. Finally, differences in magnitude in fitness values from our growth competition/Taqman assay reflected those obtained from single-cycle replication capacity assays (i.e. \( r = 0.90, P = 0.04; \) Pearson product moment). These results suggested that both methods are able to detect proportional differences between viruses with highly impaired replication capacities due to mutations in the PR and gag genes.

Comparison between ex vivo virus fitness and replication capacity of multidrug-resistant HIV-1 isolates and their pol recombinant viruses

Few studies have performed a systematic comparison of different assays to measure HIV-1 fitness (Resch et al., 2002; Prado et al., 2002; Lu & Kuritzkes, 2001) or have analysed individual contributions of different HIV-1 genes to virus fitness (e.g. mutant PR and RT) (Bleiber et al., 2001). In this study, we compared virus fitness and single-cycle replication capacity of four HIV-1 primary isolates with different patterns of drug-resistance mutations in pol (Table 2). In general, and similar to the results obtained with the APV-selected viruses, a strong correlation was observed between the fitness determined by growth competition/TaqMan and single-cycle replication capacity assays (\( r = 0.81, P = 0.04; \) Pearson product moment). However, a marked impairment on virus fitness of virus A94 (harbouring the multi-NRTI-resistance 151 mutation complex) was observed using the growth competition/TaqMan assay, while no effect in replication capacity was observed with the single-cycle assay (59 and 107 %, relative to the corresponding wild-type virus control; Fig. 5A). It is possible that mutations outside the 3′Gag/PR/RT region have influenced the virus fitness of this HIV-1 isolate, suggesting that the PR and RT regions are not solely responsible for the virus fitness of a primary HIV-1 isolate obtained from antiretroviral-treated individuals.

To test both assays, growth competition/TaqMan and single-cycle replication and to discern the contribution of different coding regions within pol to virus fitness, five sets of recombinant viruses were constructed for each one of these four drug-resistant isolates: (i) clones comprising only the PR or (ii) only the RT in a wild-type HIV-1HXB2 background, and clones harbouring the (iii) PR, (iv) RT or (v) 3′Gag/PR/RT fragment in a wild-type HIV-1NL4-3 backbone (Fig. 5B). Both growth competition and single-cycle replication capacity assays were used to measure virus fitness of HIV-1 primary isolates and these pol pseudotyped HIV-1 clones. In the wild-type F96 virus, both ex vivo virus fitness (using the HIV-1 isolate) and replication capacity (using the 3′Gag/PR/RT recombinant virus) were similar to the HIV-1 control (i.e. 96 and 102 %, respectively; Fig. 5B). However, recombinant viruses from this isolate carrying only the PR or RT showed a decrease in virus fitness, independent of the method used (Fig. 5B), suggesting that autologous PR and RT may be necessary to maintain a high degree of virus fitness. As expected, marked differences were observed when recombinant viruses from HIV-1 isolates harbouring drug-resistance mutations in the PR and/or RT genes were analysed (i.e. F98, J94 and A94; Fig. 5B). Interestingly, the highly mutated F98 HIV-1 isolate and its 3′Gag/PR/RT recombinant showed a similar decrease in virus fitness, while the PR and RT recombinants were more fit (Fig. 5B). Thus, the combination of autologous
A. Comparison of HIV-1 multidrug resistant

B. Comparison of HIV-1 isolates vs. pol recombinants

Fig. 5. (A) Relative fitness of multi-drug-resistant HIV-1 viruses using growth competition/TaqMan (HIV-1 isolates) and single-cycle replication (3′ Gag/PR/RT recombinant virus) assays. (B) Analysis of the contribution to overall fitness of different coding regions within pol. Relative fitness of HIV-1 isolates and five pol recombinant viruses in two different HIV-1 backbones (HIV-1XH2 or HIV-1NL4-3) are indicated. ND, Not determined (a PR recombinant clone in NL4-3 was not obtained for the J94 sample).

Development of high-level resistance to current antiretroviral drugs (i.e. PI and RTI) has been associated with impairment in virus fitness. Many studies have described the effect of primary drug-resistance mutations on HIV-1 fitness (reviewed by Nijhuis et al., 2001; Quinnenones-Mateu & Arts, 2001, 2002; Clavel et al., 2000) and how selection of compensatory mutations must take place in order to restore virus fitness (Berkhout, 1999; Weber et al., 2003). Although a number of these relevant amino acid substitutions may occur at as yet unrecognized sites elsewhere in the genome, most experiments on HIV-1 fitness have employed molecular clones constructed by site-directed mutagenesis or recombinant viruses carrying HIV-1 genes from patient samples (Croteau et al., 1997; Harrigan et al., 1998; Martinez-Picado et al., 1999; Robinson et al., 2000).

In this study, we have refined our growth competition assay to measure HIV-1 fitness (Quiñones-Mateu et al., 2000, 2002) using TaqMan real-time PCR technology to detect two viruses in a mixture. We compared this novel growth competition/TaqMan assay with different measurements used to determine virus fitness of both HIV-1 isolates and recombinant viruses.

Fitness of a virus is best defined in growth competition experiments (Holland et al., 1991). Our new assay involves dual infections with two different HIV-1 strains in a single culture, followed by quantification of both viruses using TaqMan real-time PCR. This technique combines the sensitivity of PCR amplification and the quantification of hybridization tests, eliminating laborious post-PCR handling of samples for quantification. Similar to the initial development of many other HIV-1-related techniques (e.g. diagnostic tests, plasma HIV-1 RNA quantification, antiretroviral drugs or vaccines), we have designed a method to specifically measure virus fitness of subtype B HIV-1 strains. Subtype B HIV-1 isolates were competed against two different non-clade B HIV-1 controls (i.e. HIV-1A-92UG029 and HIV-1AE-CMU06) in order to obtain a relative fitness value. Initial attempts using universal PCR primers and clade-specific probes in a single genomic region for all three subtypes showed a low detection level of the virus falling behind in the competition (data not shown). A preferential PCR amplification of the most fit (most abundant) virus was observed, limiting the sensitivity of the assay. To circumvent this problem, detection and quantification of both viruses in the mixture was carried out using three different subtype-specific HIV-1 primer/probe sets labelled with two different report fluorochromes (i.e. gag oligonucleotides for the subtype B query sequences and env oligonucleotides for the subtype A or CRF01_AE HIV-1 controls). All three subtype-specific TaqMan probes were able to distinguish and quantify the corresponding HIV-1 strain from a mixture with two other HIV-1 subtypes. A lower detection level (10^2–10^3 proviral DNA copies per reaction) was observed with the TaqMan detection method than with the heteroduplex tracking assay (Fig. 3). With a broad linear dynamic range of detection (10^2–10^7)
HIV-1 copies) and a strong linear correlation between the threshold cycles and the number of virus copies, this TaqMan real-time PCR assay should be useful to detect low levels of virus production in a competition, which may improve measurement of ex vivo HIV-1 fitness.

To test our assay further, we compared the virus fitness of drug-resistant HIV-1 isolates and autologous pol recombinant viruses using three different assays: (i) virus growth kinetics; (ii) growth competition cultures followed by TaqMan detection; and (iii) single-cycle replication capacity. An equal resolution of the differences in virus fitness between APV-resistant viruses was observed when growth competition experiments or single-cycle replication capacity assays were performed (Fig. 4). A similar correlation was obtained when virus fitness of HIV-1 primary isolates with different patterns of drug resistance in pol was analysed using both methods (Fig. 5). Thus, it seems possible that a considerable decrease in HIV-1 fitness due to selection and accumulation of drug-resistant mutations in pol may overcome the effect of other viral genomic regions allowing the use of 3'Gag/PR/RT recombinant viruses to estimate replicative capacity in viruses from patients treated with PI and/or RTI. Similar results have been published recently describing consistent outcomes between growth competition experiments and single-cycle replication assays (Resch et al., 2002; Prado et al., 2002). However, differences in the magnitude of the fitness loss of some PR-resistant mutants were attributed to intrinsic variations of the assay used (Resch et al., 2002).

In this study, we observed a discrepancy between growth competition/TaqMan and single-cycle replication assays when the virus fitness of an HIV-1 isolate harbouring the multi-NRTI-resistance 151 mutation complex (virus A94) was analysed (Fig. 5A). Interestingly, contradictory results have been obtained when the fitness of viruses carrying these RT mutations (i.e. 62V, 75I, 77L, 116Y and 151M) was studied using different methods. For example: (i) no effect on virus fitness, based on virus growth kinetics (Maeda et al., 1998); (ii) fitness impairment, using single infections and competition experiments (Garcia-Lerma et al., 2000); or (iii) an increase in virus fitness, comparable to the wild-type control when growth competition experiments were used (Kosalaraksa et al., 1999). All these studies analysed the virus fitness of this mutation complex in a HIV-1HXB2 backbone. Here we identified a reduction in virus fitness of the A94 virus using growth competition/TaqMan assay (HIV-1 isolate), while no effect was observed with the single-cycle assay (recombinant virus). Based on these discrepancies, it is evident that amino acid changes outside the 3’Gag/PR/RT region used in this and other studies to generate recombinant viruses may have a significant impact on the overall fitness of viruses containing the 151 mutation complex.

Finally, a previous study described the individual contribution of different HIV-1 genes to virus fitness (i.e. mutant PR and RT) (Bleiber et al., 2001). Singular recombinant clones, carrying PR, RT and PR/RT from drug-resistant virus isolates, showed a marked impairment on virus fitness using virus growth kinetic assays. However, the fitness of the parental drug-resistant HIV-1 isolates was similar to the wild-type control (Bleiber et al., 2001). In our study, we extended this analysis by comparing the virus fitness of four HIV-1 primary isolates and the contribution of PR, RT and 3’Gag/PR/RT fragments to the overall fitness in the absence of drug. Both assays, growth competition/TaqMan and single-cycle replication, generated similar results with a strong significant correlation ($r=0.72$, $P=0.01$; Pearson product moment). Even more interesting, a difference in virus fitness relative to the HIV-1 isolate was observed when recombinant viruses carrying only the PR or RT gene were analysed, including the wild-type P96 strain, stressing the importance of using HIV-1 isolates or at least the whole genomic region implicated (e.g. 3’Gag/PR/RT) to measure fitness of drug-resistant variants.

In conclusion, we have developed an ex vivo system to measure HIV-1 fitness based on growth competition experiments followed by TaqMan real-time PCR. Our results showed that both methods, growth competition/ TaqMan and single-cycle replication assay, were able to detect differences between viruses with highly impaired replication capacities due to mutations in pol and gag. However, consequent with the actual trend of multi-target antiretroviral therapies (e.g. PR, RT, integrase and entry inhibitors), this new assay may be useful to measure virus fitness of subtype B HIV-1 primary isolates or recombinant viruses carrying pol and env.

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